Title
Biochemical analysis of mRNA localization in Drosophila melanogaster and Xenopus laevis

Author
Wilhelm, James E.

Publication Date
2000

Peer reviewed|Thesis/dissertation

Biochemical Analysis of mRNA Localization in Drosophila melanogaster and Xenopus laevis

by

James E. Wilhelm

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

I dedicate this thesis to my parents, James and Janet Wilhelm,

who helped open many of the doors that led me here.

Acknowledgements

I will begin by thanking my advisor, Dr. Ronald Vale, without whom this project would probably have never seen the light of day. It is hard for me to imagine another advisor who would have allowed me to pursue so many projects that were, at best, tangential to the main interests of the lab. When I began this work, neither of us knew much about RNA localization and we spent quite some time trying to figure out what system would be best for attacking the problem. The fact that I worked on bacteriophage antitermination, *Xenopus* translational regulation, and ultimately, *Drosophila* oogenesis in my quest for a way to crack the problem, would have driven most advisors into an apoplectic fit. In fact, when I would describe what I was working on to people outside of the lab and I was invariably asked, "Why are you doing that in Ron Vale's lab?" Ron never asked this question. That faith, together with his ceaseless support in helping me find the resources and expertise I needed, made this project a reality. I would also like thank Ron for his crocodile spaghetti, his provoking Christopher to attack me on numerous occasions, his friendship, and his help in getting a job at the Carnegie.

I would also like to thank Dr. Greg Conway for helping me get started in biology when I was an undergraduate at Harvard making the transition from physics to biology. When we were in the Gilbert lab, Greg always treated me as a equal and was a lot of fun to work with. Whether we were planning on trying to purify telomerase from testicular tumors (since sperm have long telomeres), wondering if recombination occurred in the brain, or making stage-specific

libraries from Zebrafish, Greg always had great ideas and a scatological acronym to go with them.

My thesis committee also deserves much thanks: Tim Mitchison (who moved to Harvard), Dave Morgan, and Christine Guthrie. Most of my interactions with my committee actually took place outside of meetings on a fairly regular basis. Tim was a force of Nature (and Cell and Science) on our floor and was always willing to talk about all things cytoskeletal at any time. Dave was helpful when I began an ill-fated look at Exu phosphorylation and was remarkably tolerant when I would distract Hernan (see below) from his CAK purification. Christine has been great at including me in many of the social and scientific functions in the RNA world, namely their ski trips and journal clubs. Her lab also taught me yeast genetics when my project turned in that direction.

I would also like to thank my collaborator, Dr. Tulle Hazelrigg, for her amazing fly strains and even more amazing good nature. This project would have been enormously difficult without her his^6-Exu-GFP fly strains and I look forward to collaborating with her in the future.

Now it is time to thank members of the Vale lab. I would like to start with the other three horsemen of the apocalypse: Josh Niclas, Jim Hartman, and Geno de Hostos. Together, we let loose a storm of cynicism and doom that few labs have seen before or since, much less survived. A few of our innovations: the bogometer (perfect for measuring just how bogus the experiment was), the DOOR of DOOM (the real reason we moved to the 10^{th} floor), and the 3 o'clock gross-out hour (you had to be there, although you might wish you weren't). As Frank McNally once told me, "Jim, if you're this cynical starting graduate school,

I'd hate to see you when you finish." On the other hand, I think that Jim Hartman said it best in his thesis, "Hiding optimism behind a veneer of cynicism seems to be a trademark of a certain subset of Vale lab members." Of course , Jim had particular insight into this subject since we have shared the same brain for many years. Thank you, Josh, for playing Peter Gabriel in German, endless discussions on bad television, and the scary ability to complete my sentences. Thank you, Geno, for the Vale Lab Cinema showing of "El Topo," the ability to sound sarcastic no matter how sincere you are, and the discussions on the nature of karmic retribution. Thank you, Jim, for fixing everything in the lab, your appreciation that irony is the fifth basic force in the universe, and for playing Stadler to my Waldorf (the Muppets in the balcony) at many a group meeting.

At the other extreme, I would like to thank Dara Spatz Friedman for her kindness and good cheer. We are in many ways polar opposites, but she has been an unusually good friend. In spite of her inability to eat anything spicy, I will miss her greatly. Nora Hom-Booher has made the lab run smoothly for many years using her much feared wet noodle. Without her, the lab surely would have degenerated into a 10th floor version of Lord of the Flies. Ryan Case has shared the same bench with me for many years. Our long running Snood and Doom matches will be sorely missed. Sarah "Explosion Man" Rice has been a great lab mate with her love of the Matrix soundtrack and all things Ramstein. Unfortunately, ever since she hid my Grosse Point Blank soundtrack for a week (due to excessive air play), I always wonder if she has taken my missing CDs or if I have merely misplaced them. Pete Takizawa has been the other RNA localization person in the lab and has provided a great sounding board for many

of my more dubious ideas. Kurt Thorn is the current brewmaster in the lab replacing Jim Hartman. I have enjoyed our conversations on bioinformatics and biology greatly and I look forward to our continued collaboration in the future.

Special mention must be made of Manu Hegde and Vishu Lingappa. The number of stories, both true and apocryphal, surrounding these two scientists is astronomically large. Suffice it to say, I have enjoyed our countless scientific discussions on the corner of 6th and Kirkham at four in the morning and I aspire to the level of insomnia that both of you have attained. I suspect that since Manu is now at the NCI, I will continue to be caught periodically in his circadian wake when I move to Baltimore. My friendship with Hernan Espinosa and Lisa Weinberg has made the last few years a lot more enjoyable. I have enjoyed many dinners and movies with them and I will miss their companionship. Their disfunctional cat, Asta, on the other hand, I will not miss at all. Paul Peluso and I have been friends for many years after our first traumatic encounter at four in the morning. No one can eat as much, drink as much, smoke as much, or curse as much as Paul. His baleful influence has made my life a lot more colorful. I will miss him greatly. Maki Inada and Cathy Collins have broadened my horizons significantly by being my Lindy Hop partners for the last year and a half. I will miss having them to go dancing. I will also miss the not insignificant amount of verbal abuse that went along with driving them back and forth to Lindy class.

Lastly, I would like to thank my parents, James and Janet Wilhelm. I can't imagine how hard it would have been to get to this point in my life with out their constant help and support. They are two of the best people I have known in my life. My mother's kindness is truly remarkable. My father,

fortunately, has my sense of humor and has always supplied good advice. Thanks to you both and all of the people I have known here at UCSF. This was truly as special time for me.

Biochemical Analysis of mRNA Localization in *Drosophila melanogaster* and *Xenopus laevis*

by

James E. Wilhelm

Abstract

mRNA localization is used by many polarized cells as a means of restricting the distribution of a protein to a particular cytoplasmic domain. However, the molecular basis of this phenomenon is poorly understood. The identification and characterization of the components of mRNA transport complexes is the central question of this thesis. Our initial efforts focused on the development of a one-hybrid screening system for identifying proteins that bind to mRNA localization sequences. This work constituted a proof of principle for this screening method.

Since translation and localization are tightly coupled, we also investigated the translational regulation of Vg1, a localized message from *Xenopus laevis*, in an effort to understand what role the translational repression machinery might play in mRNA localization. This work has indicated that the coordination of localization with translational activation is more complicated than previously thought and that mRNAs that encode secreted proteins may pose special problems for the mRNA localization machinery.

Previous genetic studies implicated *exuperantia* (*exu*) in *bcd* mRNA localization, but its role in this process is not understood. We biochemically isolated Exu and demonstrated that it is part of a large RNAse sensitive complex that contains at least seven other proteins. One of these proteins was identified

as the cold shock domain RNA binding protein Ypsilon Schactel (Yps), which binds directly to Exu and colocalizes with Exu in both the oocyte and nurse cells of the *Drosophila* egg chamber. Surprisingly, the Exu/Yps complex contains *osk* mRNA. This biochemical result led us to re-examine the role of Exu in the localization of *osk* mRNA. We discovered that *exu* null mutants are defective in *osk* mRNA localization in both nurse cells and the oocyte. Furthermore, both Exu/Yps particles and *osk* mRNA follow a similar temporal pattern of localization in which they transiently accumulate at the oocyte anterior and subsequently localize to the posterior pole. We propose that Exu is a core component of a large protein complex involved in localizing mRNAs both within nurse cells and the developing oocyte.

Ronald Vale

Ronald Vale, PhD.
Advisor

Christine Guthrie

Christine Guthrie, Ph.D.
Committee Chair

Table of Contents

List of Tables

Chapter 1

List of Figures

Introduction

Chapter 1

Chapter 2

From: Tracey Davies <Tracey.Davies@blacksci.co.uk>

To: "'Jim Wilhelm'" <jwilhelm@cgl.ucsf.edu>

Subject: FW: Thesis permission

Date: Fri, 5 May 2000 10:03:59 +0100

Dear Mr Wihelm,

Thank you for your recent e mail requesting permission which has been passed onto myself for reply.

I can confirm that we would be happy to grant you permission to reproduce in your PhD Dissertation pp317-323 from Vol 1:3 of Genes to Cells, subject to the full acknowledgements of source.

Regards

Tracey Davies

Senior Permissions Assistant

The Rockefeller
University Press
1114 First Avenue, 4th Floor
New York, New York 10021
(212) 327-8023
Fax (212) 327-8029

May 2, 2000

Dr. Jim Wilhelm

Dear Dr. Wilhelm:

We will grant you permission for the reproduction of JCB-vol:148(3),427-440,2000-article as referred to in your letter dated May 1, 2000.

We request that you also obtain permission from the author(s) and give suitable acknowledgment to the source in the following manner: Reproduced from The Journal of Experimental Medicine, year, vol., pp. by copyright permission of The Rockefeller University Press.

Sincerely yours,

THE JOURNAL OF
EXPERIMENTAL MEDICINE

Laura L. Smith
Permissions Coordinator

Introduction

Overview

One of the central problems in cell biology is the question of how cells target proteins to specific intracellular compartments. Historically, interest in this question has focused predominantly on how proteins are targeted to membrane-bound organelles. As a result, a great deal is known about the molecular machinery responsible for targeting proteins to the nucleus, mitochondria, and the secretory pathway (Moroianu, 1999; Neupert, 1997; Rapoport, 1990).

In contrast to the wealth of information that is available concerning the sorting of proteins to membrane-bound compartments, very little is known about how proteins are partitioned within the cytoplasm. Over the last fifteen years, it has become increasingly clear that the transport of mRNAs, and not the translated proteins themselves, constitutes one of the major pathways for sorting proteins within the cytoplasm. From the initial finding that actin mRNA is unevenly distributed in the ascidian embryo (Jeffery et al., 1983), an increasing number of transcripts have been found to be localized in an ever larger variety of cell types and organisms (Bashirullah et al., 1998). While the phenomena of mRNA localization is well documented, the mechanism remains unclear due to the paucity of proteins that have been directly implicated in the transport process.

The identification and characterization of the components of mRNA transport complexes is the central question I have attempted to address in this thesis. My initial efforts focused on the development of a one-hybrid

screening system for identifying proteins that bind to mRNA localization sequences. This work is described in Chapter 1. Since translation and localization are tightly coupled, I also investigated the translational regulation of Vg1, a localized message from *Xenopus laevis*, in an effort to understand what role the translational repression machinery might play in mRNA localization. This work, described in Chapter 2, has indicated that the coordination of localization with translational activation is more complicated than previously thought and that mRNAs that encode secreted proteins may pose special problems for the mRNA localization machinery. Lastly, Chapter 3 describes my biochemical isolation of a putative mRNA transport complex from *Drosophila melanogaster* and the characterization of the protein components of the complex.

In this introduction, I will briefly review what types of messages are localized, the evidence for a step-wise localization pathway, and previous work on identifying candidate components of the transport complex.

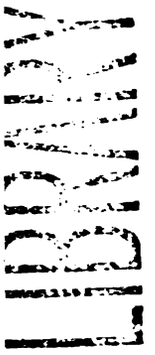
Why Sort mRNA?

mRNA localization has been most extensively characterized in *Drosophila* oogenesis where its role is to establish protein gradients that pattern the early embryo (Driever and Nusslein-Volhard, 1988; Driever and Nusslein-Volhard, 1988). *Drosophila* oogenesis takes place in a complex of 16 cells that are interconnected by cytoplasmic bridges called ring canals. 15 of these cells are nurse cells that manufacture protein and RNA for transport to the 16th cell in the complex, the oocyte. While the majority of proteins and

transcripts are merely transported to the oocyte by bulk flow, a few key regulatory molecules are actively transported to particular positions within the oocyte (Spradling, 1993). Of these localized messages, two of the best studied examples of this process are *bicoid* and *nanos*. *bicoid* mRNA is localized to the anterior pole of the oocyte and encodes a homeodomain family transcription factor that initiates a series of concentration-dependent transcriptional events that pattern the anterior of the embryo (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988; Driever and Nusslein-Volhard, 1988). *nanos* mRNA, on the other hand, is localized to the posterior pole and encodes a Zn-finger protein that promotes posterior pattern formation by blocking the translation of *hunchback*, a transcription factor induced by the *bicoid* cascade (Irish et al., 1989; Wharton and Struhl, 1991). Thus, the localization of these transcripts to opposite ends of the oocyte generates opposing gradients of these two antagonistic factors to establish the anterior-posterior body plan.

In addition to establishing protein gradients, mRNA localization is also used to generate asymmetric cell divisions by ensuring that only one cell inherits all of the mRNA encoding a particular regulatory factor. The two best studied examples of this use of mRNA localization are mother-daughter mating type switching in *Sacchromyces cerevisiae* and mesoderm induction by Vg1 in *Xenopus laevis*. In mother-daughter mating type switching, ASH1 mRNA is localized to the bud tip during mitosis to ensure that ASH1p is only found in the daughter cell (Long et al., 1997). Since ASH1p is a repressor

of mating type switching, this means that only mother cells can switch mating type. Similarly, Vg1 mRNA is localized to the vegetal pole of the Xenopus oocyte (Melton, 1987). Since the first mitotic cleavage occurs along the boundary of the animal and vegetal poles, only those cells that are descended from the initial vegetal cell will express Vg1 protein. Because Vg1 protein is a key regulator of both mesoderm induction and left-right asymmetry, the segregation of Vg1 message to the vegetal cell of the first mitotic division is crucial for establishing many cell fate decision in the embryo (Dale et al., 1993; Hyatt et al., 1996; Hyatt and Yost, 1998; Kessler and Melton, 1995; Thomsen and Melton, 1993). Thus, mRNA localization provides a powerful mechanism for ensuring that a particular protein is only expressed in one cell after mitosis has been completed.

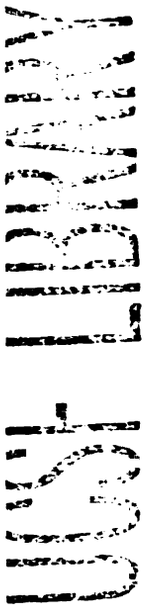


mRNA localization also provides a unique method for spatially controlling macromolecular assembly reactions. Several types of cytoskeletal proteins, such as vimentin and muscle myosin, self-assemble rapidly after translation, which necessitates restricting their synthesis to those regions of the cell where the filaments are required (Isaacs et al., 1989; Isaacs and Fulton, 1987). In the case of vimentin, the coincident changes in mRNA localization and filament distribution that occur during muscle development support the idea that mRNA localization determines the distribution of these polymers (Cripe et al., 1993). Similarly, recent experiments have demonstrated that poly(A)$^+$ mRNA and ribosomes are recruited to focal adhesion complexes that are induced by fibronectin-coated beads. Furthermore, the amount of

poly(A)$^+$ mRNA and ribosomes recruited to the focal adhesion complex increases when the beads are put under tension (Chicurel et al., 1998). These results suggest that mRNA localization may play a role in regulating or remodeling various intracellular filament systems in response to extracellular cues.

Work on the localization of β-actin mRNA also supports the idea that mRNA localization may help remodel the cell in response to external signals. β-actin mRNA is concentrated in the lamellaepodia of motile cells and the growth cones of neurons (Bassell et al., 1998; Lawrence and Singer, 1986). However, when either fibroblasts or neurons are starved for growth factors, β-actin mRNA assumes a perinuclear distribution. When serum starved fibroblasts are subsequently exposed to PDGF or lysophosphatidic acid, the β-actin message is rapidly transported to the leading edge (Latham et al., 1994). Similarly, treatment of neurons with neurotrophin-3 causes β-actin mRNA to be rapidly redistributed to the growth cone while γ-actin mRNA remains in the cell body (Zhang et al., 1999). Thus, the distrubution of β-actin mRNA is tightly regulated by external cues. Of course the critical question is whether or not these regulated localization events have any functional importance for regulating actin filament formation. Recent work with neurotrophin-3 treated neurons has attempted to address this question by examining how the distribution of actin protein changes in response to changes in β-actin mRNA localization (Zhang et al., 1999). Interestingly, β-

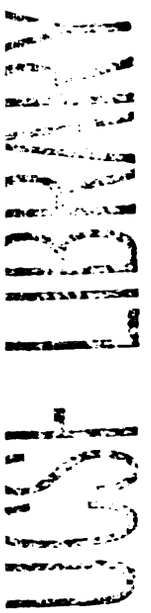
actin protein is only found in the growth cone under conditions where β-actin mRNA is localized to the growth cone (Zhang et al., 1999). Furthermore, treatment of neurons with neurotrophin-3 causes an increase in actin polymerization in the growth cone that parallels the growth cone localization of β-actin mRNA. While it is still unclear how important mRNA localization is for motility of growth cones and migrating fibroblasts, these results make it likely that mRNA localization is used to regulate the localized synthesis of actin and thus drive filament formation in both lamellaepodia and growth cones.



While all of these examples of mRNA localization clearly demonstrate the importance of partitioning cytosolic proteins, they do not explain why mRNA localization is used for protein sorting when all other forms of protein targeting rely upon protein based signals. One of the key advantages of mRNA transport is that a single mRNA can be translated many times making sorting a few mRNAs much more efficient than sorting hundreds or thousands of proteins. Another key advantage is that translation can be made dependent on proper mRNA localization thereby ensuring correct protein positioning and preventing deleterious protein-protein interactions from occurring elsewhere in the cell. mRNA localization also allows the generation of a wide variety of spatial distributions of proteins by modulating the distribution of mRNA as well as the diffusion of the translated protein from its site of synthesis. In the case of proteins such as vimentin that assemble rapidly after translation, the

distribution of protein can be very precisely defined by the localization of its mRNA (Isaacs et al., 1989). Bicoid protein, on the other hand, diffuses from its site of synthesis, thereby establishing a gradient across the *Drosophila* embryo (Driever and Nusslein-Volhard, 1988). Thus, mRNA localization affords a number of advantages over post-translational sorting for regulating protein distribution.

Step-wise assembly of mRNA transport complexes



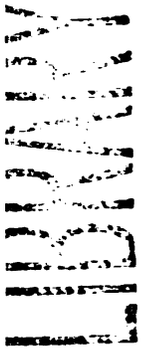
While organisms as diverse as *X. laevis, D. melanogaster, S. cerevisiae,* and *H. sapiens* all display some form of mRNA localization, the phenomenon in all of these systems displays remarkable similarities. First, virtually all of the cis-acting sequences responsible for mRNA sorting that have been mapped lie in the 3' untranslated region (3'UTR) of the message (Bashirullah et al., 1998; Kislauskis and Singer, 1992). Second, pharmacological experiments with agents that depolymerize either actin filaments or microtubules have demonstrated a requirement for these cytoskeletal elements for proper transport of mRNA (Carson et al., 1997; Pokrywka and Stephenson, 1995; Sundell and Singer, 1991; Yisraeli et al., 1989; Yisraeli et al., 1990). Third, pharmacological experiments have also demonstrated a requirement for actin filaments in maintaining a message at its site of localization (Lantz et al., 1999; Yisraeli et al., 1990). Lastly, many localized transcripts are translationally regulated to ensure that translation will only occur after the message has reached its proper destination (Gavis and Lehmann, 1994; Kim-Ha et al., 1995; Rongo et al., 1995). These

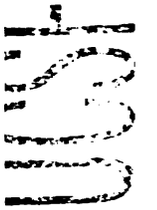
phenomenological observations all point to a simple model for mRNA transport where the cis-acting localization elements are responsible for nucleating the formation of a ribonucleoprotein complex that in turn recruits a cytoskeletal motor that transports the RNP to it correct destination. Once the mRNA reaches its final destination, it is then anchored to the cytoskeleton and activated for translation (Figure 1).

Assembly of a cytoplasmic RNA transport particle

Most biological sorting events, such as membrane trafficking, nuclear import, and protein translocation across the endoplasmic reticulum, are mediated by large macromolecular assemblies. The first indication that RNA may be transported as a large RNP particle came from studies of the BC1 message, a 152 bp RNA polymerase III transcript that is localized to the dendrites of mammalian neurons (Tiedge et al., 1991). When extracted from neuronal tissue, the BC1 RNA was discovered to be part of a 10S RNP complex (Kobayashi et al., 1991). However, the function and composition of this complex remains to be elucidated. Since BC1 is not translated and hence different from most other localized RNAs, it has been uncertain whether a large RNP complex is a universal requirement for transport. High resolution *in situ* hybridization experiments by a number of laboratories have shown that localized mRNAs display a granular pattern in the cytoplasm consistent with the presence of a transport particle containing multiple copies of transported message (Ainger et al., 1993; Cripe et al., 1993; Taneja et al., 1992). Although such observations might be discounted as

fixation artifacts, fluorescently labeled mRNA encoding myelin basic protein (MBP) also forms similar sized particles that move in a microtubule-dependent manner within a few minutes of being microinjected into oligodendrocytes (Ainger et al., 1993). Particle formation, however, is not uniquely associated with localized mRNAs, since globin mRNA also forms non-motile particles after microinjection (Ainger et al., 1993). Furthermore, the fact that each message is only labeled with one fluorophore indicates that multiple messages are being assembled into a particle since a single message is insufficiently bright to visualize. These experiments also raise the question of whether localized and nonlocalized messages are assembled into distinct complexes. Coinjection experiments with differentially labeled localized and nonlocalized messages will help address the specificity of particle assembly in this system.





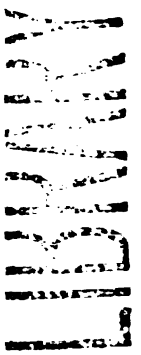
While these results argue that there is a large complex involved in transporting localized messages, the nature of this complex has remained murky. For instance, the necessity for a large RNP transport complex has been called into question by work on Vg1 mRNA localization demonstrating that the RNA binding protein vera/VgRBP specifically recognizes the Vg1 localization element and is associated with the ER (Deshler et al., 1997). This result implies that localized mRNAs may be docked to motile vesicles via membrane-associated RNA binding proteins. One of the major conclusions of Chapter 3 of this thesis is that Exuperantia, a *Drosophila* protein required for mRNA localization, is part of a large 20-60S RNAse sensitive complex

that contains the localized mRNA, *oskar*, as well as 7 additional polypeptides. This result argues quite strongly that large RNP complexes play a role in localizing mRNAs, but does not address the larger question of whether these transport complexes recruit cytoskeletal motors directly or whether they dock localized messages to transport vesicles.

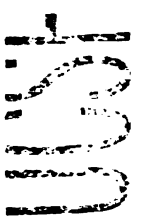
How are these large RNP complexes assembled? As mentioned previously, the specific recognition of localized messages relies on cis-acting localization sequences present in the 3'UTR. However, while the minimal region required for localization has been mapped for a number of transported messages, most analyses have found very little sequence conservation amongst these localized elements (Bashirullah et al., 1998). For example, the 3'UTRs of *bicoid* mRNAs from different *Drosophila* species all support normal mRNA localization in *Drosophila melanogaster*, even though they have diverged considerably in sequence (MacDonald, 1990). Interestingly, these *bicoid* 3'UTRs are all predicted to form a similar secondary structure, which suggests that trans-acting factors may recognize the RNA's conformation rather than its sequence (MacDonald, 1990). Obviously, the identification of the set of proteins that recognize these cis-acting localization signals is critical to understanding how mRNAs are recruited to the localization pathway as well as the mechanism of transport. Consequently, a great deal of effort has been expended trying to identify proteins that specifically recognize localization elements. Candidate localization element binding proteins have been isolated biochemically from

oligodendrocytes, *Xenopus* oocytes, and chick embryo fibroblasts (Deshler et al., 1998; Havin et al., 1998; Hoek et al., 1998; Ross et al., 1997). However, since none of these systems has tractable genetics, the role of these proteins for mRNA localization *in vivo* has been difficult to address. Also, the fact that the proteins that have been isolated, such as hnRNP A1 in oligodendrocytes and hnRNP I (polypyrimidine tract binding protein) in *Xenopus*, are believed to play a role in a number of RNA regulatory events makes genetic analysis one of the few ways to determine whether these proteins are contributing specifically to mRNA localization (Cote et al., 1999; Hoek et al., 1998). The recent use of sensitized substrates and temperature sensitive alleles to demonstrate that the hnRNP-like protein, Hrp1p/Nab4p, in yeast plays a specific role in nonsense-mediated mRNA decay shows how a combination of genetics and biochemistry can be used to tease specific functions out of the seemingly promiscuous hnRNP proteins (Gonzalez et al., 2000). The work on Hrp1/Nab4 also points to a possible role for nuclear history in influencing the fate of mRNA in the cytoplasm. This idea is also supported by the recent finding that the correct localization of microinjected *ftz* transcripts in *Drosophila* embryos only occurs if the message has been preincubated in a nuclear extract prior to microinjection (Lall et al., 1999). This work, which also demonstrated that the hnRNPA1-homologue, *squid*, crosslinks to the *ftz* message, indicates that the nuclear history is important for making a message competent to be localized (Lall et al., 1999). One interpretation of this work is that some of the specifc RNA recognition

events occur in the nucleus. However, it is equally plausible that the role of proteins, such as hnRNPA1, is to act as "chaperones" for assembly of the transport complex by stabilizing the correct conformation of the localization element so that the components of the transport machinery can efficiently assemble on the localization element. This view is supported by the fact that cytoplasmically injected MBP mRNA and Vg1 mRNA are both transported to their respective destinations without having passed through the nucleus (Melton, 1987; Ainger et al., 1993). One way around these issues is to focus on purifying the assembled transport complex from the cytoplasm and then confirming the functional relevance of each component of the complex through genetic analysis. This is the experimental approach that was used to dissect *oskar* mRNA localization in Chapter 3.



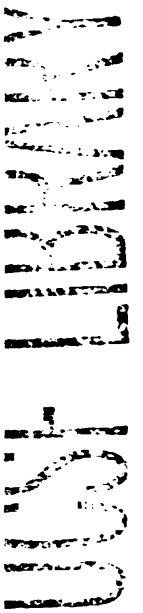
The role of the cytoskeleton in cytoplasmic mRNA transport



The first indications that the cytoskeleton was required for proper transport of mRNA came from studies in a number of systems that demonstrated that cytoskeletal inhibitors block mRNA transport. Interestingly, not all mRNA localization events are blocked by the same classes of cytoskeletal inhibitors. For instance, the transport of *bicoid* mRNA is very sensitive to agents that depolymerize microtubules, but is insensitive to actin depolymerizing agents (Pokrywka and Stephenson, 1994). Conversely, β-actin mRNA localization is highly senstive to actin depolymerizing agents and insensitive to microtubule depolymerizing drugs (Sundell and Singer, 1991). These findings were the first to suggest that both

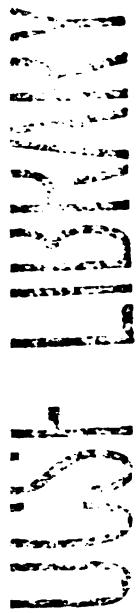
actin and microtubules play a role in the transport process and that the filament system used is transcript specific.

These experiments were further buttressed by work in *Drosophila* with mutations that cause disruptions in the polarity of the microtubule array such that the minus-ends of microtubules are at both the anterior and posterior poles while the plus-ends of microtubules are in the center of the oocyte. In these mutants, *bicoid* mRNA accumulates at both the anterior and posterior poles, while *oskar* mRNA accumulates in the center of the oocyte (Micklem et al., 1997). This result argued strongly for an active transport process for both *oskar* and *bicoid* transcripts that relied on the polarity of the microtubule network to sort mRNAs to their proper locations. While both the pharmacological and genetic experiments strongly indicated an active role for the cytoskeleton in mRNA transport, it was still formally possible that the cytoskeleton played an indirect role in the transport process.



This issue was definitively addressed by experiments in both oligodendrocytes and *S. cerevisiae* where mRNA transport was visualized directly by tagging the localized transcript with a fluorophore. While the techniques used for visualization in each of these experiments were different, the conclusions were the same: mRNA localization is an active process along cytoskeletal filaments. In oligodendrocytes, the unidirectional movement of MBP mRNA occured at speeds constent with a microtubule-based motor and was dependent on the presence of microtubules (Ainger et *al.,* 1993). Even more strikingly, the transport of ASH1 mRNA particles into

the daughter cell required both actin filaments and the type V myosin, MYO4/SHE1p (Long et al., 1997; Takizawa et al., 1997). Furthermore, MYO4/SHE1p coimmunoprecipitates with ASH1 mRNA and colocalizes with ASH1 mRNA *in vivo* (Bertrand et al., 1998; Long et al., 1997; Takizawa et al., 1997). Thus, MYO4/SHE1p is the first cytoskeletal motor directly implicated in an mRNA transport event. This finding, together with the results from oligodenrocytes, support the model that cytoskeletal motor proteins, such as myosin, kinesin, and dynein, are responsible for transporting mRNAs to their final destinations. However, the identity of these motor proteins and the mechanisms that are used to recruit them to the transport particle remain unknown. The isolation and biochemical characterization of mRNA transport complexes combined with the development of *in vitro* motility assays for mRNA transport should help resolve these issues.

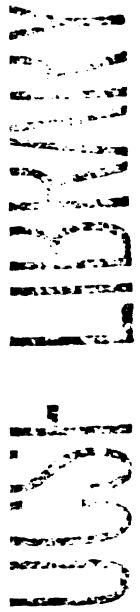


Anchoring of localized mRNAs

After transport is completed, the localized mRNA must maintain its final distribution. The active transport process that initially localized the mRNA could be used to collect the RNA that diffuses away from its final destination. However, microtubule inhibitors, which abolish active transport of mRNA in oocytes, fail to disperse localized Vg1 or *bicoid* mRNA (Pokrywka and Stephenson, 1991; Yisraeli et al., 1989; Yisraeli et al., 1990). Furthermore, the reorganization of microtubules at stage 10 of *Drosophila* oogenesis is not accompanied by a corresponding redistribution of localized

messages (Theurkauf et al., 1992). These results argue that mRNAs become anchored at their final destination by a mechanism independent of microtubules and cytoplasmic transport.

Some element of the cytoskeleton is almost certainly involved in anchoring messages, since localized mRNAs, in contrast to other RNAs, are not solublized by Triton X-100 (Yisraeli et al., 1990). Actin filaments are the most likely candidates, since Vg1 becomes dispersed after cytochalasin treatment (Yisraeli et al., 1990). Cytokeratins have also been suggested to participate in RNA retention (Pondel and King, 1988), but their role is probably secondary to actin's, since fragmentation and disassembly of cytokeratins in oocytes does not release the Vg1 transcript from the detergent-insoluble matrix (Klymkowsky et al., 1991).



The anchoring of transcripts to the cytoskeleton presents another opportunity for the cell to regulate mRNA distribution. The localized Vg1 message, for instance, is found initially in the detergent-insoluble cytoskeletal fraction, but then becomes detergent soluble at the time of oocyte maturation (Pondel and King, 1988; Yisraeli et al., 1989; Yisraeli et al., 1990). This change in detergent extractability occurs at the time that Vg1 message loses its tight cortical localization and becomes diffusely distributed over the vegetal hemisphere (Melton, 1987). In contrast, the vegetally localized XCAT-2 transcript remains in the detergent insoluble fraction throughout oogenesis (Mosquera et al., 1993). Thus, the cytoskeletal associations of

different localized mRNAs can be controlled independent of one another.

Coordinating mRNA Translation with Localization

To ensure a highly restricted protein distribution, it is generally thought that mRNA translation is repressed during transport and then activated upon arrival at its destination. The dependence of translation on proper mRNA localization prevents the synthesis of proteins from transcripts that are either en route to their destination or that have become mislocalized. This may be particularly important in oocytes, where translation of mislocalized mRNAs could have deleterious effects on embryogenesis.

The first support for this idea came from work on the *oskar* and *nanos* mRNAs which found that mutations that block mRNA localization also block the translation of these messages. Since this initial observation, the RNA elements that mediate translational repression have been found to lie within the 3'UTR and overlap with the sequences that mediate localization (Bergsten and Gavis, 1999; Kim-Ha et al., 1995). The proximity of these sequence elements has suggested that the coordination of localization with translational activation may be due to the fact that anchoring the transcript dislodges the repression machinery since they share overlapping binding sites (Bergsten and Gavis, 1999). However, more recently work with *oskar* mRNA has shown that while sequences in the 3'UTR are required for translational repression, there are sequences in the 5'UTR that are required for translational activation (Gunkel et al., 1998). Thus, the mechanism of

coordinating localization and with translation is likely to be more complicated than simple competitive binding to a regulatory element.

One simple way for regulating translation is by altering the length of the poly(A) tail by cytoplasmic polyadenylation. This mechanism for translational repression of maternal transcripts has been previously described for the c-mos mRNA in *Xenopus* and *bicoid* mRNA in *Drosophila* (Salles et al., 1994; Sheets et al., 1994). In both of these cases, the transcript has an unusually short poly(A) tail that is lengthed upon fertilization allowing the message to be translated. However, for *nanos* and *oskar* mRNA there are no apparent changes in poly(A) length associated with translational activation (Gavis et al., 1996; Lie and Macdonald, 1999; Lieberfarb et al., 1996). Furthermore, recent work on reconstituting *oskar* mRNA translational repression *in vitro* has shown that the translational repression is not relieved by the addition of excess free cap analog implying that 5′ cap recognition is not the step of translation that blocked (Lie and Macdonald, 1999). In work described in Chapter 2, we have found that the localized *Xenopus* transcript Vg1 is also translationally repressed until localization is completed and that this repression is is independent of the length of the poly(A) tail. Thus, the phenomenon of localization-dependent translational regulation is not unique to *Drosophila* and is independent of poly(A) tail length in both systems.

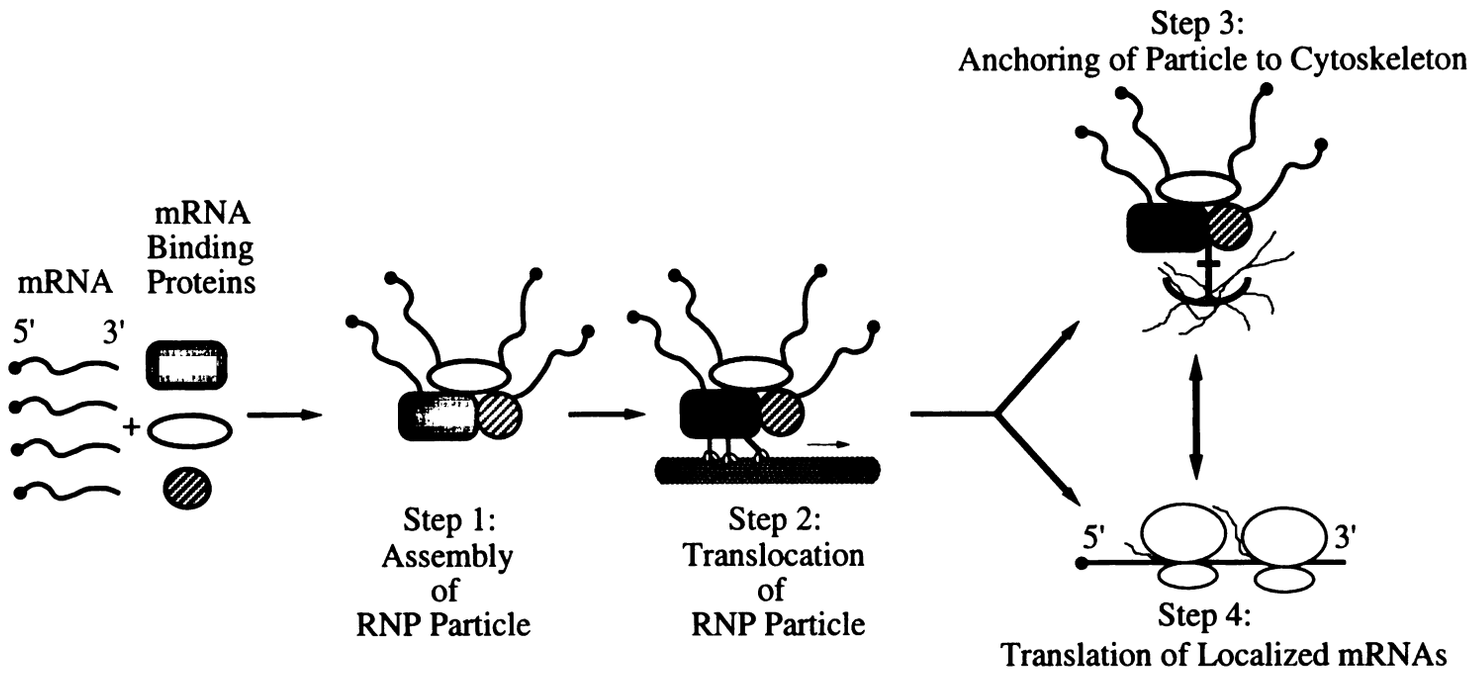
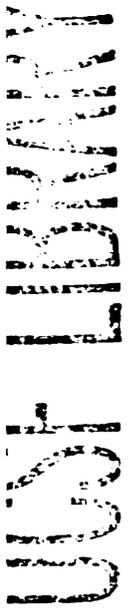


Figure 1: A multi-step model for mRNA transport. Step 1: The localized message is assembled into a ribonucleoprotein (RNP) complex. Step 2: The RNP particle recruits cytoskeletal motors and commences translocation along the cytoskeleton. Step 3: The RNP particle is anchored to the cytoskeleton once transport is complete in order to insure stable localization. Step 4: Translation of the localized mRNA.



Chapter 1

Abstract

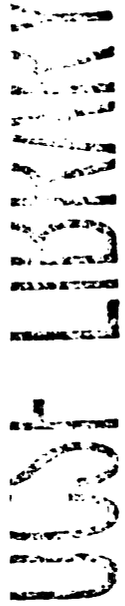
The two-hybrid system has greatly facilitated the study of protein-protein interactions. Here we describe a bacterial one-hybrid system for studying RNA-protein interactions that exploits the ability of the bacteriophage lambda RNA binding protein, N, to cause transcription antitermination. Our results show that fusion of a heterologous RNA binding protein to N causes antitermination and β-galactosidase expression in a reporter construct that has the heterologous binding site substituted for the normal N target sequence. This reporter system is capable of discriminating between RNA-protein interactions of differing affinities and is sufficiently sensitive to detect interactions with K_ds as weak as 10^{-5} M. The system should be useful for identifying novel proteins that bind RNA regulatory sites.

Introduction

The bacteriophage lambda gene product N is a small sequence specific RNA binding protein that is required for expression of both the rightward and leftward early operons. N protein positively regulates bacteriophage lambda early gene expression by binding to and modifying RNA polymerase in a manner that allows it to read through transcription termination signals (Das, 1993; Greenblatt et al., 1993). Assembly of the antitermination complex requires a cis-acting RNA sequence called the Nut site which contains two distinct sequence elements: boxB, which binds N, and boxA, which interacts with the host accessory factors NusB and S10 (Franklin, 1985; Nodwell and Greenblatt, 1991; Nodwell and Greenblatt, 1993). A variety of experiments have revealed that N binds to boxB through a short N-terminal region, while the remainder of the protein appears to interact with RNA polymerase and the host factor, NusA (Greenblatt and Li, 1981; Lazinski et al., 1989; Whalen et al., 1988). Such findings suggested to us that the N-mediated antitermination system may be modular and could be exploited to detect heterologous RNA-protein interactions.

To explore this idea, we examined whether the N protein system could be used to detect a well characterized RNA-protein interaction- the binding of the R17 coat protein to its operator site (Carey et al., 1983; Lowary and Uhlenbeck, 1987; Romaniuk et al., 1987; Schneider et al., 1992). R17 coat protein is a gene product of the RNA phage R17 which is involved in packaging the R17 genome into the phage particle and repressing translation of the R17 replicase gene. This RNA-protein interaction was chosen as a test case for our investigation, because the binding affinities of the R17 coat protein for several operator site mutants have been determined (Romaniuk et al., 1987; Schneider et al., 1992).

In this study, we have shown that a fusion protein consisting of R17 coat protein joined to N causes antitermination in a reporter plasmid in which boxB of the Nut site has been replaced with the R17 operator. The efficiency of antitermination depends upon the affinity of the R17-operator interaction. This system could be used to study known RNA-protein interactions or identify novel proteins that bind to RNA regulatory sites.



Results

R17-N Fusion Proteins are Capable of Antiterminating Through a Composite R17-boxA Site

The principles underlying the function of the reporter system and how this system can be modified to detect RNA-protein interactions are diagrammed in Fig. 1. The unmodified reporter for detecting N function consists of a Nut site followed by 4 terminators, and then by the LacZ gene (Figs. 1a, 2b). In the absence of a second plasmid supplying N protein, transcription terminates prior to reaching the LacZ gene and β-galactosidase is not produced. However, when N is supplied by a second plasmid, RNA polymerase can read through the terminators and β-galactosidase is expressed, thereby providing a quantitative measure of antitermination (Fig. 1a,b). In order to determine whether antitermination can occur through a site that is not normally recognized by N, we modified this reporter system by replacing boxB of the Nut site with the R17 operator.

To evaluate the specificity of this system, the ability of N protein to cause antitermination at an R17-boxA target site was examined. N protein co-expressed with a reporter containing an R17-boxA target site generated only 1-2 units of β-galactosidase activity, in contrast to the 900 units of β-galactosidase activity produced from a boxB-boxA (nut site) containing reporter. Thus, N protein alone cannot assemble an antitermination complex at an R17-boxA site. In order to target N protein to the R17-boxA site, a fusion protein was constructed in which R17 was joined to the N-terminus of N protein though a linker peptide consisting of 5 glycine residues (R17-N; Fig. 2a). When the R17-N fusion protein was tested with the R17-boxA reporter plasmid, a 3-4 fold increase in β-galactosidase expression was observed over a reporter construct lacking the R17-boxA site

(Table 1). This increase in β-galactosidase expression also yielded a clear difference in colony color on X-gal indicator plates (Fig. 3). R17 expressed alone, without being fused to N, did not produce antitermination (data not shown). Similarly, the R17-N fusion protein did not cause increased β-galactosidase expression with a reporter that had an R17 site without boxA, indicating that host cell factors are required for antitermination (data not shown).

A Modified Reporter System for Detecting Low Affinity RNA-Protein Interactions

To examine the sensitivity of the system, an R17 operator containing a point mutation that lowers its affinity for R17 coat protein ($R17^{low}$; K_d of 10^{-5} M compared to 10^{-8} M for the wild type site (Romaniuk et al., 1987)) was examined (Fig. 2c). No appreciable difference in either β-galactosidase activity (Table 1) or colony color (Fig. 3) was observed between the $R17^{low}$-boxA site and the reporter construct lacking the binding site. One possible reason for the inability to detect this low affinity RNA-protein interactions was that the antitermination complex dissociated from the nascent transcript before the polymerase reached the terminators. If this were true, then decreasing the distance between the R17-boxA site and the terminators might increase the sensitivity of the assay. To test this idea, 1.5 kb of DNA that lay between the target site and the terminators was deleted (pDel; Fig. 2b). As shown in Table 1, the pDel reporter construct containing the R17-boxA target site exhibited a significant increase in signal-to-background compared to the parent pTAT reporter. More importantly, it became possible to detect the interaction of R17-N with the $R17^{low}$-boxA site by both colony color and β-galactosidase assays (Table 1;Fig. 3). Differences in β-galactosidase expression

between the wild type R17 operator and a high affinity ($K_d = 10^{-10}$ M) R17 operator (Fig. 2c), however, were difficult to discern with either the pDel or pTAT plasmids (data not shown), possibly because in this range of dissociation constants, the binding of R17-N to the RNA was no longer the limiting step for the formation of an antitermination complex.

Fusions of R17 to the Carboxy Terminus of N Protein

Although the endogenous RNA binding domain of N is located at the amino terminus, we wanted to determine whether or not R17 could be fused to the carboxy terminus of N and still preserve the antitermination functions of N. As shown in Table 1 and Fig. 3, fusion of R17 to the C-terminus of N through a 5 glycine linker (N-R17) also caused antitermination through the R17-boxA or R17^{low}-boxA target sites. Although the absolute signal was lower, the signal to background ratio was similar to the N-terminal fusion, and colonies containing the R17^{low}-boxA target could be easily distinguished from those that did not by their color on X-gal plates (Fig. 3). The C-terminal fusion, however, had difficulty resolving between the R17-boxA and R17^{low}-boxA target sites (Table 1) for reasons that are unclear. The efficiency and the ability to discriminate between different affinity binding sites potentially could be improved by modifying the peptide linker between N and R17.

Discussion

In this study, we have demonstrated a one-hybrid system that should be a useful tool for studying RNA-protein interactions, since it can detect low affinity interactions and can discern between equilibrium dissociation constants in the range of 10^{-5} to 10^{-8} M. Furthermore, our findings with the pDel and pTAT reporter plasmids suggest that increasing or decreasing the distance between the target site and the terminators may modulate the dynamic range of the system and thereby make it possible to detect differences in RNA-protein affinity over a wide range of K_ds.

The ability to detect interactions on the basis of bacterial colony color suggests that this technique can be applied to screening libraries for proteins that bind to a known RNA sequence. Such a screening system may have advantages over other methods for studying RNA-protein interactions that are based upon TAT or REV function in mammalian cell lines or that use translational interference as a reporter system (MacWilliams et al., 1993; McDonald et al., 1992; Selby and Peterlin, 1990; Stripecke et al., 1994; Tiley et al., 1992; Venkatesan et al., 1992). A library screening system using N-mediated antitermination as a reporter takes advantage of *E. coli*'s facile molecular genetics, which should make isolation and re-testing of positives substantially easier than in mammalian systems. Furthermore, our system generates a positive signal (i.e. transcription of a reporter gene) which allows even weak binding events to be easily distinguished from background, whereas other systems rely on a loss of signal to detect an RNA-protein interaction (Stripecke et al., 1994).

The finding that N protein can trigger antitermination through a heterologous RNA-protein interaction also sheds some light on the antitermination mechanism. The RNA binding domain of N protein is a 25

amino acid arginine-rich peptide that is similar to the arginine rich RNA binding domains of several of other proteins, including TAT and REV from human immunodeficiency virus (HIV) (Kjems et al., 1992; Lazinski et al., 1989; Weeks et al., 1990). Structural studies of the arginine-rich domain of TAT have shown that it undergoes a pronounced conformational change upon binding to it's target sequence, TAR, in the HIV genome (Calnan et al., 1991). Since the RNA binding domains of N protein and TAT are closely related, one might have anticipated that N also undergoes a conformational change upon binding boxB that is required for antitermination. However, the fact that R17, which binds RNA via a β-sheet motif (Valegard et al., 1994), can be fused to N protein and can cause antitermination through the R17 operator argues that such conformational changes are not absolutely required. Rather, RNA binding may be involved primarily in tethering N and increasing its effective concentration in the vicinity of RNA polymerase, as suggested by *in vitro* studies of antitermination (Mason et al., 1992; Nodwell and Greenblatt, 1991).

Our observation that antitermination through a low affinity R17 site can be made more efficient by decreasing the distance between the binding site and the terminators also suggests that complex stability is a key determinant in achieving long distance antitermination. This *in vivo* result complements previous *in vitro* experiments that have suggested that host cell accessory factors create a network of protein-protein interactions that stabilize that antitermination complex and allow it to act at long distances (Mason et al., 1992). By using low affinity RNA-protein interactions to target N to the nascent transcript (such as R17-N binding to R17^{low}-boxA) and modulating the levels of accessory factors, it may be possible to dissect the *in vivo* role of the accessory factors in achieving long distance antitermination.

Acknowledgments

We would like to thank Naomi Franklin for generously providing plasmids for this study. J.E.W. acknowledges support from the Medical Scientist Training Program. This work was supported, in part, from a grant from the National Institutes of Health.

Materials and Methods

Bacterial Strains and Assays for Antitermination

The bacterial strain N567 (Franklin, 1993; Franklin, 1989), was made competent for transformation (Chung et al., 1989), and then co-transformed with the indicated reporter and activator plasmids. Co-transformants were selected on plates containing chloramphenicol (15 mg/l) and ampicillin (50 mg/l).

β-galactosidase activity was assayed on plates containing chloramphenicol (15 mg/l), ampicillin (50 mg/l), 0.025 mM IPTG, and 0.17 mM X-gal. Plates were incubated at 37°C for 20-24 hr and then colony color was examined. For liquid assays, overnight cultures were diluted 50-fold into LB containing 50 mg/l ampicillin and 15 mg/l chloramphenicol, grown until OD_{600} = 0.2 and then induced with 0.5 mM IPTG for 1.75 hr. Cells were permeabilized with SDS/chloroform and assayed for β-galactosidase activity using o-Nitrophenyl-β-D-galactoside (ONPG) as a substrate (Miller, 1992)

Construction of Reporter and Activator Plasmids

Reporters containing either the R17-boxA or $R17^{low}$-boxA binding sites were constructed by altering the pTAT13 reporter construct (generously provided by N. Franklin) by using partially overlapping oligonucleotides that

were annealed and then extended with Klenow fragment of DNA polymerase (Madhani and Guthrie, 1994). The resulting DNA fragment was digested near the ends with SalI and BamHI and then inserted into the SalI/BamHI sites of pTAT13. In order to prevent translation from starting at the Shine-Dalgarno sequence encoded by the R17 operator, the operator sequence was altered at two positions. The first mutation in the Shine-Dalgarno sequence, which changed the first GC below the loop to CG, does not alter the binding affinity of the coat protein for the site (Romaniuk et al., 1987). The second mutation changed an AU base pair in the stem to a UA basepair, and thereby removed the initiation codon associated with the Shine-Dalgarno sequence. *In vitro* RNA selection experiments have shown that the coat protein does not prefer an AU over a UA at this position (Schneider et al., 1992).

pDel reporters were generated by digesting pTAT13 with XbaI and KpnI, blunting the ends with Klenow fragment of DNA polymerase, and then recircularizing the plasmid. This procedure removed the 1.5 kb PhoA gene from the pTAT13 reporters.

Fusions of R17 to N protein were constructed in the expression plasmid pBR-ptac N* (Franklin, 1993). Fusion of R17 to the amino terminus of N protein was done by using PCR to generate an R17 fragment that had flanking NcoI sites and a 5 glycine linker attached to the amino terminus of R17. This fragment was ligated into NcoI digested pBR-ptac N* to generate the R17-N construct (N-terminal fusion). Fusion of R17 to the carboxy terminus of N was accomplished by using PCR to generate a ClaI/BamHI fragment of R17 that had 5 glycines attached to the amino terminus of R17. This fragment was ligated into ClaI/BamHI digested pBR-ptac N* to generate the N-R17 construct (C-terminal fusion).

Table 1: Quantitation of β-galactosidase for various reporter/activator combinations

Activator	Reporter	Binding Site	Alk. Phos.Units (Mean ± S.D.)	β-Gal Units (Mean ± S.D.)	Percent of Control
N protein	pTAT	*nut* site	1348 ± 18	947 ± 78	—
	pTAT	No site	699 ± 73	1.8 ± 0.3	Control
	pTAT	*boxA*-R17	869 ± 20	1.6 ± 0.1	100
	pDel	No site	N/A	1.2 ± 0.03	Control
	pDel	*boxA*-R17	N/A	<1	100
R17-N protein	pTAT	No site	1340 ± 103	20 ± 2	Control
	pTAT	*boxA*-R17^{low}	778 ± 25	29 ± 4	145
	pTAT	*boxA*-R17	863 ± 51	75 ± 5	375
	pDel	No site	N/A	20 ± 1	Control
	pDel	*boxA*-R17^{low}	N/A	47 ± 2	235
	pDel	*boxA*-R17	N/A	141 ± 23	705
N-R17 Protein	pDel	No site	N/A	4.4 ± 0.2	Control
	pDel	*boxA*-R17^{low}	N/A	16 ± 1	364
	pDel	*boxA*-R17	N/A	12 ± 1.5	273

Values for β-galactosidase activity were determined as described in the Materials and Methods and are the mean of assays conducted in triplicate. Assays on reporters in the absence of the activator plasmid all yielded less than 1 unit of Beta-Galactosidase activity. N/A is not applicable.

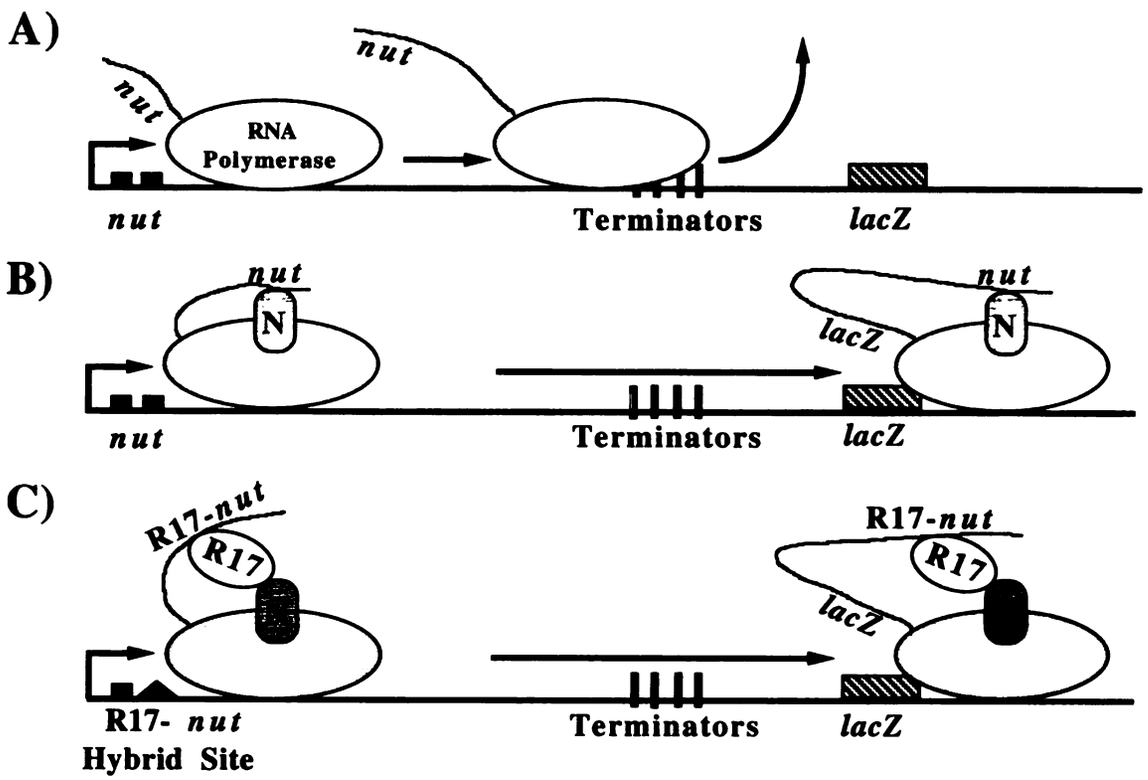
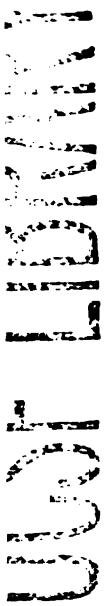
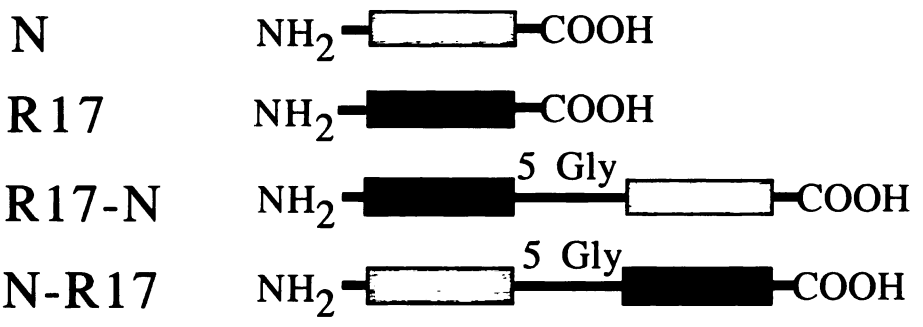

A)
nut
nut
RNA
Polymerase
nut
Terminators
lacZ
B)
nut
N
nut
lacZ
N
nut
Terminators
lacZ
C)
R17-nut
R17
R17-nut
R17
lacZ
R17- nut
Hybrid Site
Terminators
lacZ

Figure 1: Assays For N-mediated Antitermination and Detection of Heterologous RNA-Protein Interaction. A) RNA polymerase transcribes the *nut* site, but in the absence of N protein, transcription is terminated prior to reaching *lacZ*. B) RNA polymerase transcribes the *nut* site and N protein binds the nascent transcript. N, in conjunction with cellular factors that are not shown, then modifies the polymerase, which enables it to read through the terminators and transcribe *lacZ*. C) To detect a heterologous RNA-Protein interaction, *boxB* of the *nut* site is replaced with the R17 operator sequence. An R17-N fusion protein recognizes the hybrid site which triggers antitermination and transcription of *lacZ*.

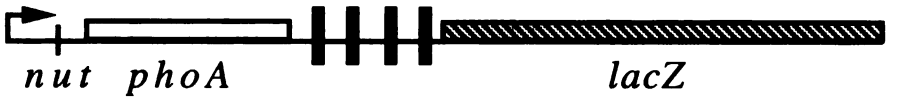


A) Expressed Proteins

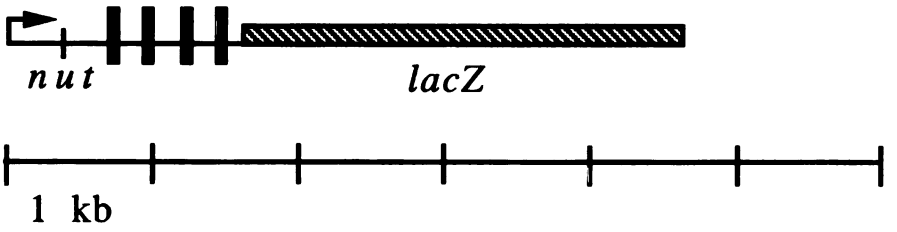


B) Reporter Constructs

pTAT



pDel



C) Hybrid R17-*nut* Site

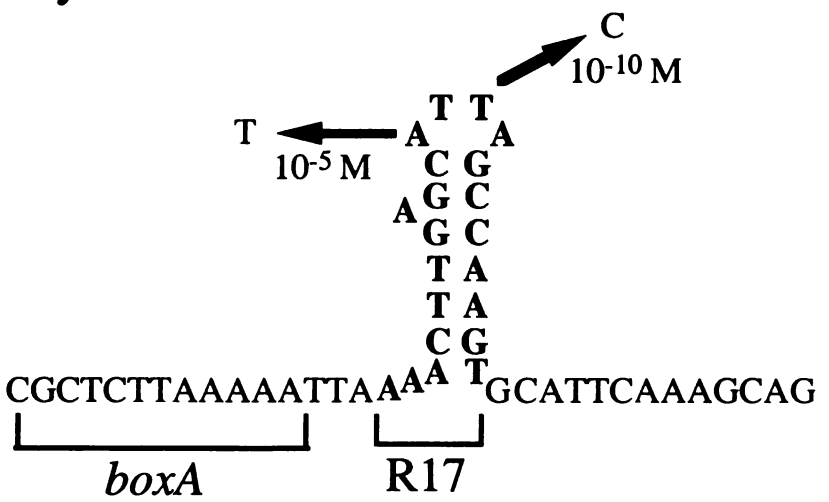


Figure 2: Diagrams of Expressed Proteins and Reporter Constructs.

A) Representation of the various expressed proteins tested in the antitermination assay. B) pTAT plasmid and the modified pDel plasmid, which has the 1.5 kb *phoA* gene deleted, were used to detect low affinity RNA-protein interactions. The transcription start site is indicated by an arrow and the transcription terminators are indicated by black bars. Each plasmid is diagrammed with the nut site present. However, for R17 experiments this site was replaced with various *boxA*-R17 target sites shown in panel C. C) Sequence and proposed secondary structure of the hybrid *boxA*-R17 site used to test R17-N fusions for antitermination activity. The stem-loop of the R17 binding site is indicated in bold. The affinity of R17 coat protein for the wild-type R17 site shown is 10^{-8} M. The point mutants that were used are indicated with arrows.

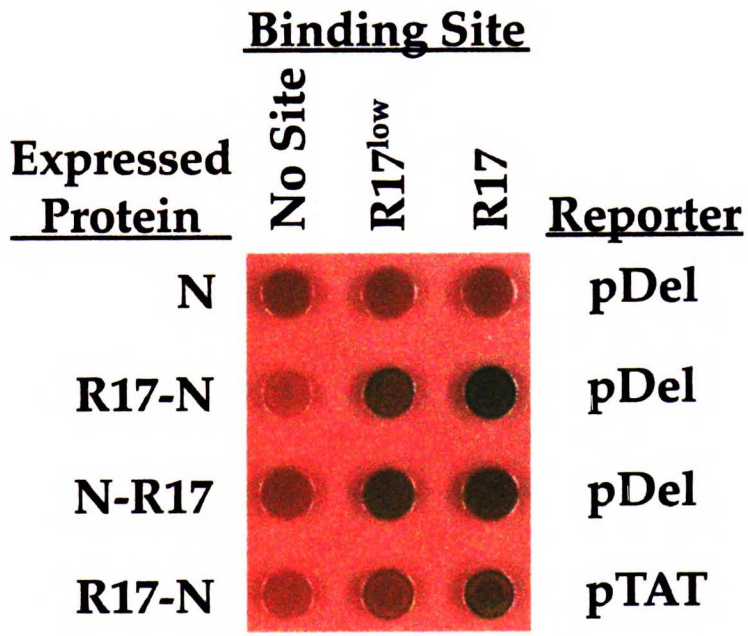

Binding Site
No Site
R17low
R17
Expressed Protein
Reporter
N
pDel
R17-N
pDel
N-R17
pDel
R17-N
pTAT

Figure 3: Detection of Heterologous RNA-Protein Interactions by Colony Color on an X-gal/IPTG Plate. The top row indicates the binding site present in the reporter construct. The left column indicates the activator protein that was expressed. The right column indicates whether the reporter plasmid was pTAT or pDel. The colony color was developed as described in the Materials and Methods.

Chapter 2

Abstract

Vg1, a member of the TGF-β family involved in mesoderm induction, is translated subsequent to the localization of its mRNA to the vegetal pole of *Xenopus* oocytes. While the localization of Vg1 mRNA is known to be directed by the 3′ untranslated region (UTR), the basis of its translational regulation is unknown. We show here that the 3′UTR of Vg1 causes translational repression of two different reporter mRNAs. A 350 nucleotide region of the 3'UTR, which is distinct from the localization element, is necessary and sufficient for mediating translational repression and specifically binds to a 38 kD polypeptide. The translational repression activity is found throughout the oocyte and at all stages of oogenesis. These results suggest that factors colocalized with Vg1 mRNA at the vegetal pole relieve translational repression to allow expression of Vg1 protein.

Keywords: 3' Untranslated Region/ mRNA Localization/ Oogenesis/ Translational Control/ Vg1

Introduction

Specification of cell fates in the developing embryo requires the coordinated spatial and temporal expression of multiple regulatory factors. One way in which this regulation is accomplished is by localizing mRNAs encoding important regulatory molecules to particular regions of the developing oocyte (Berleth et al., 1988; Ephrussi et al., 1991; Lehmann and Nusslein-Volhard, 1991; Melton, 1987). The localization of mRNAs allows the developing oocyte and embryo to generate asymmetric distributions of proteins encoded by localized transcripts by virtue of the fact that the proteins are most highly enriched in those regions where their messages are found. These early mRNA sorting events form the basis for determining cell fate and pattern formation in a number of organisms (Bashirullah et al., 1998). However, in order for mRNA localization to achieve a high degree of fidelity, it is important to have mechanisms to prevent the premature translation of mRNAs prior to their arrival at their final destination (Bergsten and Gavis, 1999; Rongo et al., 1995).

Such a phenomenon of localization-dependent translation has been described for both the *oskar* and *nanos* transcripts in *Drosophila melanogaster* (Gavis and Lehmann, 1994; Kim-Ha et al., 1995). In these examples, the processes of localization and translational control are both controlled by sequences in their respective 3′ untranslated regions (UTRs). Moreover, the translational control elements lie within the regions of the 3′UTR that mediate localization (Gavis et al., 1996; Gavis et al., 1996; Kim-Ha

et al., 1995; Kim-Ha et al., 1993). These results have led to a model where the localization machinery competes for the same binding sites as the machinery involved in translational repression (Bergsten and Gavis, 1999). According to this view, assembly of the localization complexes on the 3'UTR relieves translational repression, thereby coupling proper localization with expression. Whether this paradigm is universally used during early developmental regulation of other transcripts remains unclear.

In *Xenopus laevis*, the TGF-β family member Vg1 is regulated both at the level of mRNA localization and translation during early development (Dale et al., 1989; Melton, 1987; Rebagliati et al., 1985; Tannahill and Melton, 1989). This maternally supplied transcript is localized in a microtubule-dependent manner to the vegetal pole during stages III and IV of oogenesis (Yisraeli and Melton, 1988; Yisraeli et al., 1990). Only after the localization of Vg1 mRNA has been completed in stage IV can Vg1 protein expression be detected for the first time (Dale et al., 1989; Tannahill and Melton, 1989). The temporal correlation between the completion of Vg1 mRNA localization and the onset of Vg1 translation suggests that these two events are coordinately regulated. In contrast to the mRNAs in *Drosophila*, relatively little is known about the mechanism of localization-dependent translational activation of Vg1 mRNA. The 3' UTR of Vg1 contains a 340 nucleotide element that is necessary and sufficient to direct its vegetal pole localization (Mowry and Melton, 1992). Recently, two RNA binding proteins, vera/VgRBP and VgRBP60, have been identified that bind specifically to the Vg1 localization

element (Cote et al., 1999; Deshler et al., 1998; Havin et al., 1998). However, the role of these proteins in localization, and their relationship to translational regulation remains unknown.

In this study, we examine the translational control of the Vg1 mRNA. We find that the 3'UTR of Vg1 mRNA mediates its translational repression. In contrast to *nanos* and *oskar* mRNAs, the translational control element (TCE) in the 3'UTR of Vg1 lies outside of the previously defined localization element. The TCE is necessary and sufficient for repression and specifically binds to a 38 kD protein. Surprisingly, the translational repression activity is present and equally distributed throughout the oocyte even in stages when the endogenous Vg1 mRNA is localized and actively being translated. Together, these results suggest that additional factors localized to the vegetal pole are responsible for relieving translational repression of Vg1 mRNA once it is correctly localized. Our work indicates that localization-dependent translational control may be a common theme in early development, and suggests a mechanism by which mislocalized Vg1 message is translationally repressed. Such regulation may be crucial in imparting fidelity to the proper spatial expression of this important regulatory molecule.

Results

The observation that Vg1 translation begins coincident with the completion of Vg1 mRNA localization in stage IV suggests that these two events may be coordinately regulated (Dale et al., 1989; Tannahill and Melton, 1989). One mechanism to achieve this coordination would be to regulate the repression machinery so that it is active during the early stages of oogenesis and then inactivated at stage IV, when Vg1 protein begins to be synthesized. Alternatively, the translational repression machinery could be actively maintained, with the Vg1 message being transported into a protected environment. We wished to address whether the Vg1 message is under direct translational control, and if so, what mechanisms might operate to coordinate its expression with localization.

Because translational control of mRNA is often mediated by sequences in the 3' untranslated region, we wished to determine whether the 3'UTR of Vg1 could also mediate translational regulation. To do this, we assessed the ability of the Vg1 3'UTR to influence the translation of other coding regions. We prepared reporter constructs consisting of either the prolactin coding sequence with no 3'UTR (Prl) or the prolactin coding sequence followed by the Vg1 3'UTR (Prl-Vg1). Both Prl and Prl-Vg1 transcripts translated equally well in a wheat germ *in vitro* translation system (Fig. 1A). In contrast, the translational efficiency of the Prl-Vg1 mRNA was markedly reduced relative to that of Prl mRNA when injected into either early (II/III) or late (V/VI) stage *Xenopus* oocytes (Fig 1B). This

repression was sequence specific, since neither a non-specific 3'UTR (consisting of the sp64 vector backbone) nor the prolactin 3'UTR produced this effect when fused to the prolactin coding sequence (data not shown). Moreover, the effects of Vg1 3'UTR appear to be reporter independent, since fusion of the Vg1 3'UTR to another reporter mRNA, β-lactamase, also caused translational repression (data not shown).

Translational repression of Prl-Vg1 could be overcome by microinjecting increasing amounts of mRNA. A ~10 fold reduction in 3'UTR specific translational repression was observed when the microinjected transcript concentration was increased from 4 to 100 nM (Fig. 1D), which suggests that the translational repression machinery is limiting. The range of RNA concentrations in which translational repression is detected is comparable to the physiologic concentration of the Vg1 message in the oocyte, and only begins to saturate at mRNA concentrations that are approximately 20 fold above the concentration of the endogenous Vg1 message (Rebagliati et al., 1985). Together, these experiments demonstrate that the 3'UTR of Vg1 is sufficient to confer translational repression in a heterologous context. Moreover, this translational repression appears to involve oocyte-specific factors, as it was not observed in an *in vitro* translation system (Fig. 1A). Thus, it appears that within the context of the oocyte, the 3'UTR of Vg1 mRNA is involved in its translational control.

Several mechanisms of 3'UTR mediated translational control have been described previously. These include altering mRNA stability (Binder et al.,

1994), changing poly(A) tail length (Sheets et al., 1994), or recruiting sequence specific RNA binding proteins that promote translational repression (Ostareck et al., 1994). To investigate whether differential mRNA stability is involved in Vg1 3'UTR mediated repression, Prl and Prl-Vg1 transcripts were microinjected into stage VI oocytes and total RNA was isolated 0, 2, and 4 hr later. We were unable to observe a systematic or significant difference between the rates of RNA degradation (Fig. 2A), arguing against differential RNA stability as the cause of the 10 fold difference observed in their protein expression.

To investigate the role of polyadenylation in Vg1 translational repression, we examined the effect of adding a synthetic poly(A)$_{20}$ tail to Prl and Prl-Vg1. The translational efficiency of both transcripts was independent of the presence or absence of the poly(A) tail (Fig. 2B). The finding that deletions at the 3' end of the UTR still result in repression (Fig. 4; described below), together with the absence of detectable size differences in transcript length over time (Fig. 2A), also argue against cytoplasmic polyadenylation as the mechanism of translational repression. The failure of mRNA stability or polyadenylation to explain the lower protein expression of Prl-Vg1 suggests that translational repression may operate via the recruitment of a protein complex to the 3'UTR. Additionally, such a translational repression machinery is predicted to be present throughout oogenesis based on the results of Figure 1B.

The finding that injected Prl-Vg1 transcripts are translationally repressed in late stage (V/VI) oocytes is somewhat surprising given that the endogenous Vg1 message is being actively translated at this time (Fig 1C). This raises the possibility that the endogenous, localized Vg1 message is in an environment that is free from repression. One way in which this might occur is through a spatial gradient of translational repression activity, with the lowest levels of repression being found in the vegetal pole. Alternatively, translational repression activity may be uniformly distributed, but factors that are colocalized with Vg1 at the vegetal pole may alleviate the repression. To distinguish between these possibilities, we compared the extent to which Prl-Vg1 is repressed in the animal versus vegetal hemispheres. Repression of the Prl-Vg1 mRNA was found to be equal in the animal and vegetal halves of stage V/VI oocytes (Fig. 3). Although the possibility of highly regional differences in translational repression activity cannot be excluded, this result argues against the existence of a global gradient of translational repression. Thus, it is possible that factors colocalizing at the vegetal pole with Vg1 relieve the message of its translational repression. This is similar to models proposed for *nanos* in *Drosophila*, where the localization machinery itself relieves the repression of transported messages (Bergsten and Gavis, 1999).

To further explore the idea that localization and translation of Vg1 are coupled by competing machineries for common binding sites, we mapped the region of the Vg1 3'UTR that is involved in translational repression.

Deletion mutagenesis coupled with subsequent functional analyses for repression revealed a 348 nucleotide element, which we term the translation control element (TCE), that is both necessary and sufficient for translational repression (Fig. 4). Interestingly, the TCE is distinct from the region of the 3'UTR that was previously determined to mediate localization (Mowry and Melton, 1992). Thus, translational repression and localization of the Vg1 mRNA involve distinct RNA recognition elements, and hence, may be mediated by distinct factors.

In order to identify proteins that might be involved in translational repression, [^{32}P]-labeled RNAs encoding different regions of the 3'UTR were added to a cytosolic oocyte extract and then subjected to ultraviolet irradiation to crosslink proteins bound to the RNA. A 38 kD protein bound and crosslinked to RNAs containing the TCE in a saturable manner, but bound with very low affinity to RNAs lacking the TCE (Fig. 5A,B). Two other prominent polypeptides of 45 kD and 55 kD were also observed, but seemed to crosslink to all regions of the 3'UTR and may represent nonspecific RNA binding proteins (Fig. 5). Consistent with this interpretation, immunoprecipitation experiments have identified the 55 kD crosslinked polypeptide as FRGY2, a general RNA binding protein (data not shown). In contrast, the binding of the 38 kD protein to the TCE was specific, and competed by a 50-fold molar excess of unlabeled TCE, but not by transcripts encoding globin or the localization element (Fig. 4C). Thus, the 38

Discussion

In this study, we have demonstrated that the Vg1 3'UTR contains a translational control element that is physically distinct from the Vg1 localization element and that specifically binds a 38 kD protein. The translational repression mediated by this element is saturable and is present in both the animal and vegetal hemispheres of the oocyte. Furthermore, the repression activity is present during all stages of oogenesis, including those where the endogenous Vg1 message is being actively translated, and is not due to changes in polyadenylation or message stability. Thus, Vg1 likely represents the first example of localization-dependent translation identified outside of *Drosophila melanogaster*.

Our data suggest a model for the coordinate control of translation and localization (Fig. 6). In this model, a homogeneously distributed repressor maintains Vg1 mRNA in a translationally repressed state during stages I and II of oogenesis. During stages III and IV, the Vg1 transcripts are recruited into transport complexes and localized to the vegetal cortex where they are anchored to elements of the cytoskeleton. Either the act of assembling the transport complex or docking the Vg1 mRNA to the cytoskeleton could functionally displace the repressor and allow Vg1 protein to be synthesized. We favor the latter alternative for two reasons. First, repression and localization are mediated by distinct and separable regions of the 3'UTR, potentially allowing localization to occur without interfering with translational repression. Second, translational repression may serve to

prevent Vg1 mRNA, which encodes a secretory protein, from being docked at sites of protein translocation at the endoplasmic reticulum (ER) during localization.

Interactions with the translocation machinery could be particularly disruptive to the localization process given the observation that the localization element of Vg1 interacts with an ER associated protein implicated in localization (vera/VgRBP) (Deshler et al., 1997). Thus, translational repression may serve to promote Vg1 mRNA localization by ensuring that Vg1 mRNA is free to target only to the portions of the ER undergoing transport via vera/VgRBP. Prevention of premature or promiscuous interactions with the ER may also be important for *gurken*, a secreted TGF-α family member whose mRNA is localized to the anterodorsal region of the *Drosophila* oocyte. In this case, the localization element is found in the 5'UTR of the message, a situation that is unique among all known localized messages (Saunders and Cohen, 1999). Assembly of a localization complex at the 5'UTR would present an obstacle to translation, and may be the mechanism by which translation and targeting of the message to the ER is prevented during localization. Thus, in contrast to mRNAs encoding cytosolic factors, it may be particularly important for messages encoding secreted proteins to be translationally repressed *during* localization.

Many of the conclusions from our work are consistent with recent findings on the translational regulation of localized mRNAs during

Drosophila development. Both *oskar* and *nanos* mRNAs are not translated if their localization is disrupted, indicating the presence of a translational repressor whose action is alleviated by the proper localization of the transcript (Gavis and Lehmann, 1994; Kim-Ha et al., 1995; Rongo et al., 1995). In the case of *nanos*, a 120 kD protein, Smaug, has recently been identified that is required for translational repression of *nanos* mRNA (Dahanukar and Wharton, 1996). In the case of *oskar*, an 80 kD RNA binding protein, Bruno, has been shown to be required both for translational repression *in vivo* and proper pattern formation (Kim-Ha et al., 1995; Webster et al., 1997). The candidate 38 kD translational repressor of Vg1 is unlikely to be the *Xenopus* homologue of Smaug or Bruno because of the molecular weight differences between the proteins. Furthermore, there do not appear to be Bruno or Smaug binding sites in the TCE of Vg1 (data not shown). This suggests that distinct translational repressors operate upon different transcripts, or that the repression machinery may differ between organisms. Yet, it appears that the coupling of translational activation to proper mRNA localization in order to prevent the precocious expression of key regulatory proteins is a conserved theme in the development of both *Xenopus* and *Drosophila*. The use of localization-dependent translational activation in both of these organisms most likely reflects a common need to generate very tight protein gradients with minimal leakage; a goal that is most easily accomplished by strongly repressing translation until a message has reached its proper destination.

Acknowledgements.

The authors would like to thank V. Lingappa for support and guidance, members of the Vale Lab for critical reading of the manuscript, and C. Zimmerman and A. Calayag for excellent technical assistance. We also thank D. Melton for providing constructs and antibodies to Vg1 and M. Murray for providing antibodies to FRGY2. JEW and RSH were supported by Medical Scientist Training Program grant GM 07618.

Materials and Methods

Construction of plasmids.

The sp64 derived bovine prolactin expression construct (BPI), which contains an SP6 promoter and the 5'UTR from *Xenopus* globin preceding the prolactin coding region, has been described previously (Andrews et al., 1989). The Prl-Vg1 construct was engineered as follows: a plasmid containing the full Vg1 3'UTR (Mowry and Melton, 1992) was digested with BstE2, treated with Klenow fragment, digested with EcoR1, and this fragment was ligated into the BPI construct that had been digested with Blp1, treated with Klenow fragment, and digested with EcoR1. The resulting plasmid was digested with EcoR1, and oligonucleotides encoding a poly(A)$_{20}$ tail were inserted to generate Prl-Vg1. The control Prl construct was made by digesting Prl-Vg1 with Spe1 and EcoR1, treating with Klenow fragment, and recircularizing the plasmid. Prl and Prl-Vg1 were linearized at either Xba1 (located just after the A$_{20}$ sequence; Fig. 2, 3) or EcoR1 (located just before the A$_{20}$ sequence; Fig. 1, and as indicated in the legends) prior to transcription. Construct C2 was made by digesting Prl-C2 with Hind3 and Spe1, treating with Klenow, and recircularizing the plasmid. C3 was made by digesting Prl-Vg1 with Hind3 and Bsm1, treating with Klenow, and recircularizing the plasmid. C7 was made by ligating a PCR fragment encoding the Vg1 3'UTR from the Bsm1 to Nsi1 sites into Prl-Vg1 digested with Hind3 and EcoR1. C8 was made by digesting Prl-C8 with Hind3 and Spe1, treating with Klenow, and

recircularizing the plasmid. The resulting plasmids were linearized at EcoR1 prior to transcription.

Cell free transcription and translation.

Cell free transcription was performed using SP6 RNA polymerase (New England Biolabs) as described previously (Andrews et al., 1989). Quantitation of RNA yield in our standard transcription reactions (40°C for 60 min) was performed by measuring the incorporation of [^{32}P]-UTP and did not vary significantly from one experiment to another. Preparation of wheat germ extract, and *in vitro* translation using this extract were as previously described (Andrews et al., 1989).

Xenopus oocyte expression.

Oocytes were removed from *Xenopus laevis* frogs and maintained at 18°C in modified Barth's saline (MBSH) (Heasman et al., 1991). They were used within 5 days of harvest. Oocytes were staged using published criteria, and transcripts microinjected into the vegetal hemisphere as indicated in the figure legends. It is important to note that RNAs containing the Vg1 3'UTR are only localized when injected into oocytes of stages I-III, and cultured through at least stage IV. RNA injected into stage II/III oocytes, but not cultured, or injected into stage V/VI oocytes, will not be localized (Yisraeli and Melton, 1988). Thus, microinjected RNAs in the experiments described in this study are free to diffuse thorughout the oocyte. Labeling was performed by incubation (at 18°C) in MBSH containing between 250-1000 μCi/ml [^{35}S]-methionine for various times as indicated in the figure legends.

Where unspecified, labeling was for 2 hrs. Following labeling, the oocytes were washed once in MBSH to remove free label, and homogenized in 50 volumes of harvest buffer (HB: 1% SDS, 0.1 M Tris-HCl, pH 8.9). The samples were heated to 100°C for 5 min, clarified by centrifugation at 12,000 x g for 2 min to remove any insoluble material, and the samples processed for immunoprecipitation with anti-prolactin (United States Biochemical) or anti-Vg1 antibodies as described previously (Hegde and Lingappa, 1996). The microinjected RNA concentration in the oocyte was estimated using an injection volume of 50 nl, a stage VI oocyte volume of 1000 nl (Smith et al., 1991), and assuming significant diffusion of the RNA during the two hr incubation period before the labeling. Thus, in Fig. 1D, the RNA concentration within the oocyte is estimated to be 20 fold less than the injected concentration. Endogenous Vg1 mRNA concentation was estimated based on previous work (Rebagliati et al., 1985) which determined that it consisted of approximately 0.05-0.1% of the 50-100 ng of poly(A) RNA per oocyte.

UV crosslinking of RNA.

Collagenased oocytes were washed thoroughly and dounce homogenized in an equal volume of ice cold homogenization buffer (0.25 M sucrose, 25 mM Hepes, pH 7.4, 50 mM KCl, 1 mM MgAcetate, 0.1 mM EDTA, 2 mM PMSF, 0.2 mM benzamidine, 5 μg/ml aprotinin, 10 μM leupeptin, 1 mM DTT), centrifuged at 10,000 x g for 10 min in a TLA 100.3 rotor (Beckman), and the supernatant recentrifuged at 200,000 x g for 40 min. This high speed

supernatant was used at a final protein concentration of 0.75 mg/ml in the UV crosslinking reactions. [^{32}P]-labeled RNA encoding the various constructs was synthesized as previously described (Schwartz et al., 1992). In instances where the absolute amount of transcript was determined, [^{35}S]-UTP labelled RNA was synthesized (Schwartz et al., 1992). The transcription reaction was then loaded on a Sepharose G-50 micro spin column (Pharmacia) in order to separate the free nucleotide from the RNA. The flow-through was phenol extracted and then ethanol precipitated overnight. An aliquot of the resuspended RNA was counted in a scintillation counter in order to determine the percent incorporation of radioactive nucleotide. The percent incorporation was used to estimate the moles of transcript synthesized.

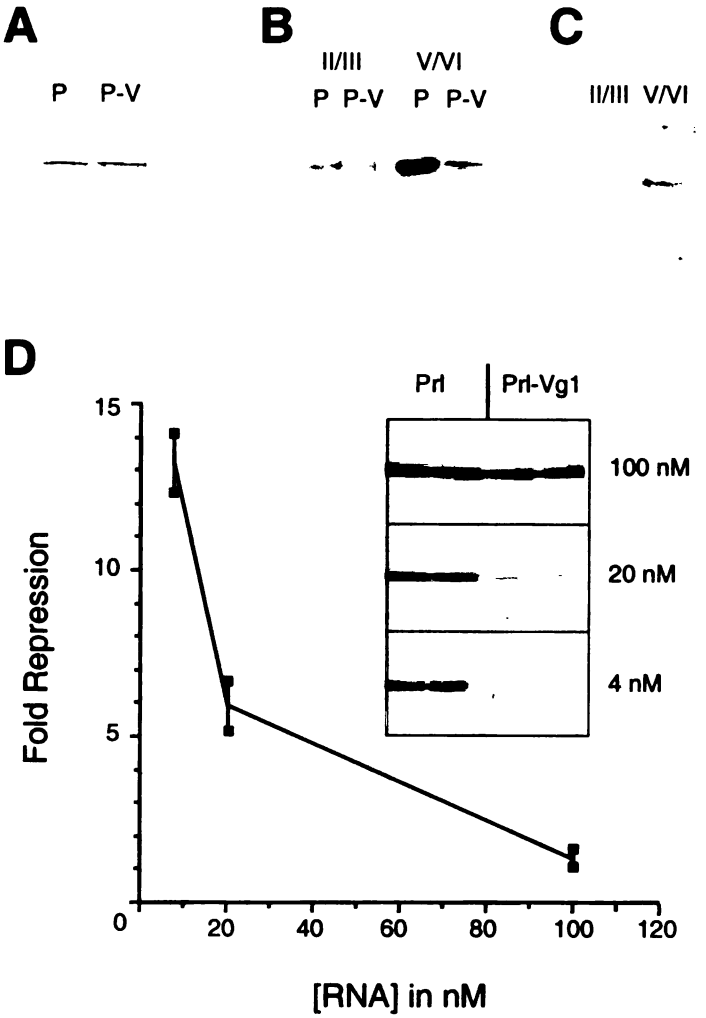
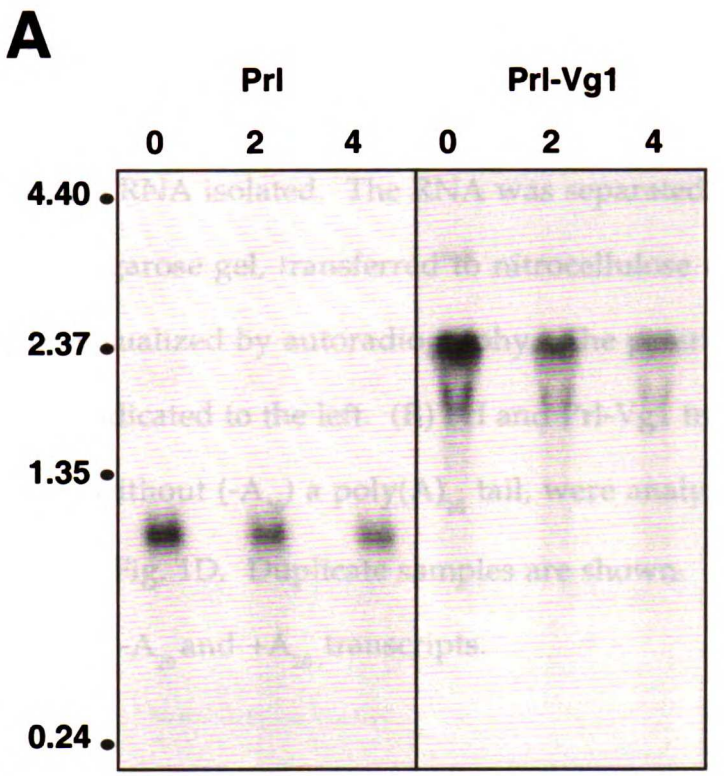

A
P P-V
B
II/III V/VI
P P-V P P-V
C
II/III V/VI
D
Prl
Prl-Vg1
100 nM
20 nM
4 nM
Fold Repression
15
10
5
0
20
40
60
80
100
120
[RNA] in nM

Figure 1. Vg1 3'UTR mediates translational repression of heterologous mRNA. (**A**) Plasmids encoding either prolactin (Prl, lane P) or prolactin followed by the 1.2 kb 3'UTR from Vg1 (Prl-Vg1, lane P-V,) were used to generate transcripts which were translated in a wheat germ extract containing [^{35}S]-methionine. Equal amounts of the translation reactions were separated by SDS-PAGE and the newly synthesized proteins were visualized by autoradiography. (**B**) Transcripts of Prl (P) and Prl-Vg1 (P-V) were injected into stage II/III (~10 nl/oocyte) or stage V/VI (~50 nl/oocyte) oocytes. Shown are the immunoprecipitates analyzed by SDS-PAGE and autoradiography. (**C**) Western blot analysis for Vg1 expression in early (II/III) and late (V/VI) stage *Xenopus* oocytes. Ovaries were removed from *Xenopus laevis* frogs and the oocytes isolated and staged. Oocytes were solubilized the same as for immunoprecipitation and the proteins in each supernatant equivalent to 0.6 oocytes (early staged sample) and 0.1 oocytes (late staged sample) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Vg1 antibodies. (**D**) Translational repression is saturable. Prl and Prl-Vg1 transcripts at an approximate concentration of 400 nM were diluted to 4, 20, and 100 nM in water prior to injection into stage VI oocytes (~50 nl/oocyte). The oocytes were incubated for two hrs at 18°C to allow the transcripts to diffuse throughout the oocyte, then labeled for two hrs in modified Barth's saline (MBSH) containing 500 μCi/ml [^{35}S]-methionine. After labeling, prolactin was immunoprecipitated. The inset shows the autoradiogram of duplicate samples for Prl and Prl-Vg1

at the three different RNA concentrations injected into the oocytes. The fold repression was determined following quantitation of the bands by densitometry, and graphed versus RNA concentration.



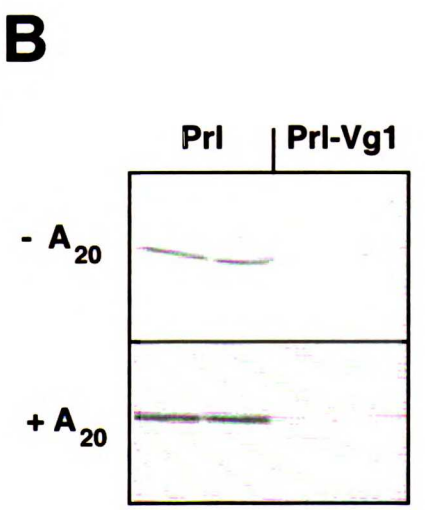


Figure 2. Characterization of Vg1 3'UTR mediated translational repression.

(**A**) Translational repression is not due to mRNA stability. [^{32}P]-UTP labeled Prl and Prl-Vg1 transcripts were microinjected into stage VI labeled transcripts oocytes, incubated for the times (hr) indicated above each lane, and total oocyte RNA isolated. The RNA was separated on a 1% formaldehyde-agarose gel, transferred to nitrocellulose membranes, and the labeled RNA visualized by autoradiography. The positions of RNA standards are indicated to the left. (**B**) Prl and Prl-Vg1 transcripts at 20 nM, with ($+A_{20}$) or without ($-A_{20}$) a poly(A)$_{20}$ tail, were analyzed for translational repression as in Fig. 1D. Duplicate samples are shown. The repression was ~ 5.5 fold for both $-A_{20}$ and $+A_{20}$ transcripts.

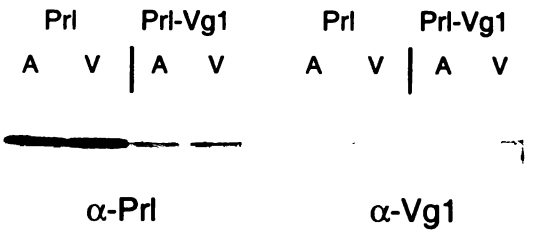
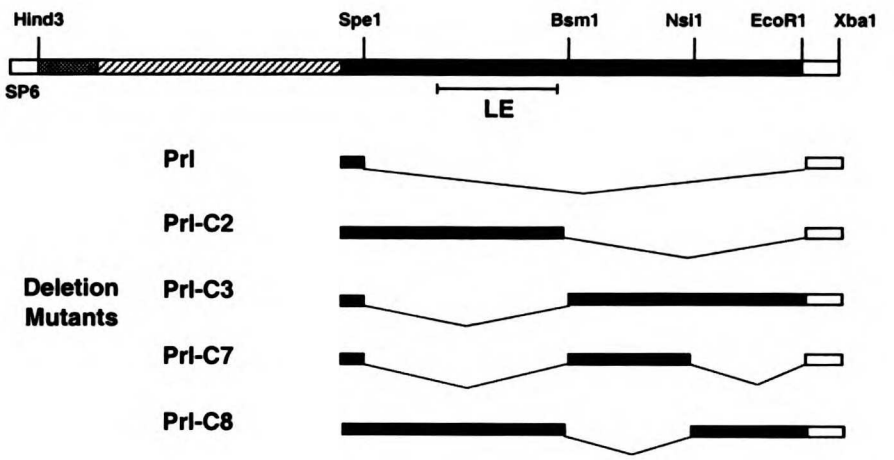

Prl
Prl-Vg1
Prl
Prl-Vg1
A
V
A
V
A
V
A
V
α-Prl
α-Vg1

Figure 3. Translational repression activity is evenly distributed throughout the oocyte. Prl and Prl-Vg1 transcripts at approximately 20 nM were injected into stage VI oocytes (50 nl/oocyte) and the oocytes incubated in MBSH for 12 hours at 18°C to ensure complete diffusion of the RNA within the oocytes. They were then labeled for 60 min with MBSH containing 1 mCi/ml [^{35}S]-methionine, fixed, and then manually dissected into animal (A) and vegetal (V) halves. Either prolactin or Vg1 was then immunoprecipitated from each half and analyzed by SDS-PAGE and autoradiography.

A



B

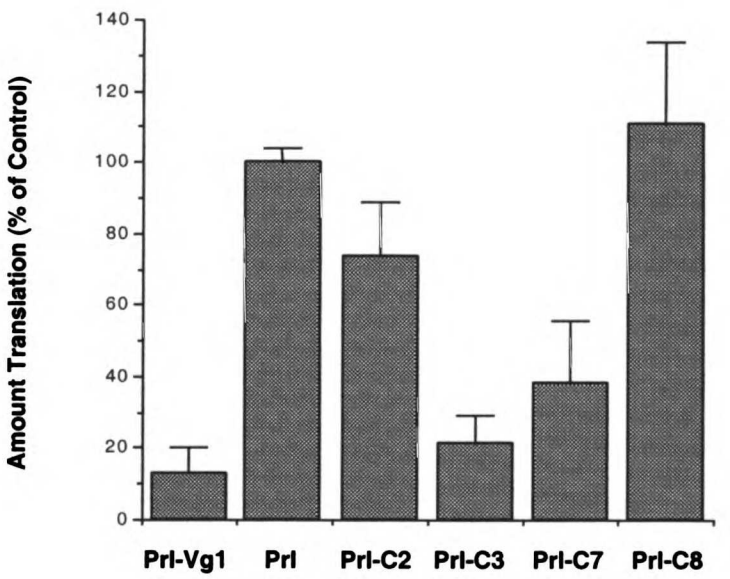


Figure 4. The translational control element (TCE) is distinct from the mRNA localization element. (**A**) Diagram of the constructs used to map the TCE. The SP6 promoter, 5'UTR (shaded), coding region (hatched), 3'UTR (black), localization element (LE), poly$(A)_{20}$ tail (white), and relevant restriction sites are indicated on the Prl-Vg1 construct. Prl, Prl-C2, Prl-C3, and Prl-C8 have deletions from Spe1 to EcoR1, Bsm1 to EcoR1, Spe1 to Bsm1, and Bsm1 to Nsi1, respectively, as diagrammed. Prl-C7 contains two deletions, from Spe1 to Bsm1 and from Nsi1 to EcoR1. (**B**) The relative translational efficiencies of the constructs diagrammed in panel A were determined (at the RNA concentration of 20 nM) as described in Fig. 1D. Values are the average of at least three measurements; standard errors are indicated by the error bars.

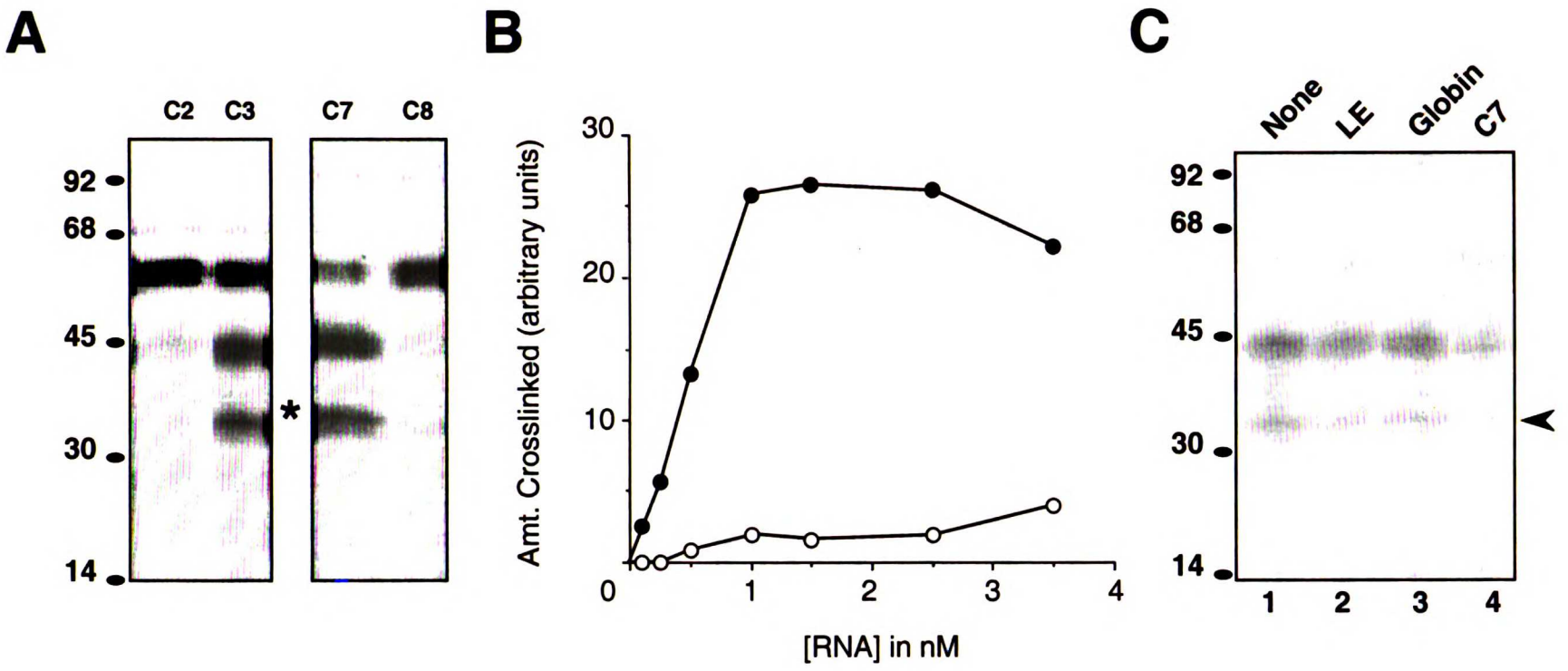

A
C2
C3
C7
C8
92
68
45
30
14
*
B
Amt. Crosslinked (arbitrary units)
30
20
10
0
1
2
3
4
[RNA] in nM
C
None
LE
Globin
C7
92
68
45
30
14
1
2
3
4

Figure 5. Crosslinking of proteins specific to the TCE. **(A)** The constructs C2, C3, C7, and C8, which correspond to Prl-C2, Prl-C3, Prl-C7 and Prl-C8, respectively, but lack the 5'UTR and coding regions were used to synthesize [^{32}P]-labeled RNA. An amount of each RNA corresponding to equal incorporated counts was incubated with *Xenopus* oocyte extract and subjected to ultraviolet (UV) mediated crosslinking. The labeled proteins were separated by SDS-PAGE and visualized by autoradiography. The arrowhead indicates the position of a 38 kD protein that appears to be specifically crosslinked to RNAs containing the TCE. **(B)** Effect of substrate RNA titration on crosslinking. Various concentrations of [^{32}P]-labeled C2 (open circles) and C3 (closed circles) RNA were used for UV crosslinking as in Panel A. The 38 kD band was quantitated by densitometry (with local background subtraction) and plotted as a function of RNA concentration. **(C)** Binding of 38 kD protein is competed by the TCE, but not by the minimum localization element or globin. RNA corresponding to the globin coding region, localization element (LE), or TCE (C7) were synthesized and quantitated. A 50 fold excess of each was mixed with [^{32}P]-labeled C7 RNA (0.25 nM final concentration) and UV crosslinking was performed as in panel A. The arrow indicates the position of the 38 kD crosslink. Lane 1 is the crosslinking reaction containing no competitor RNA.

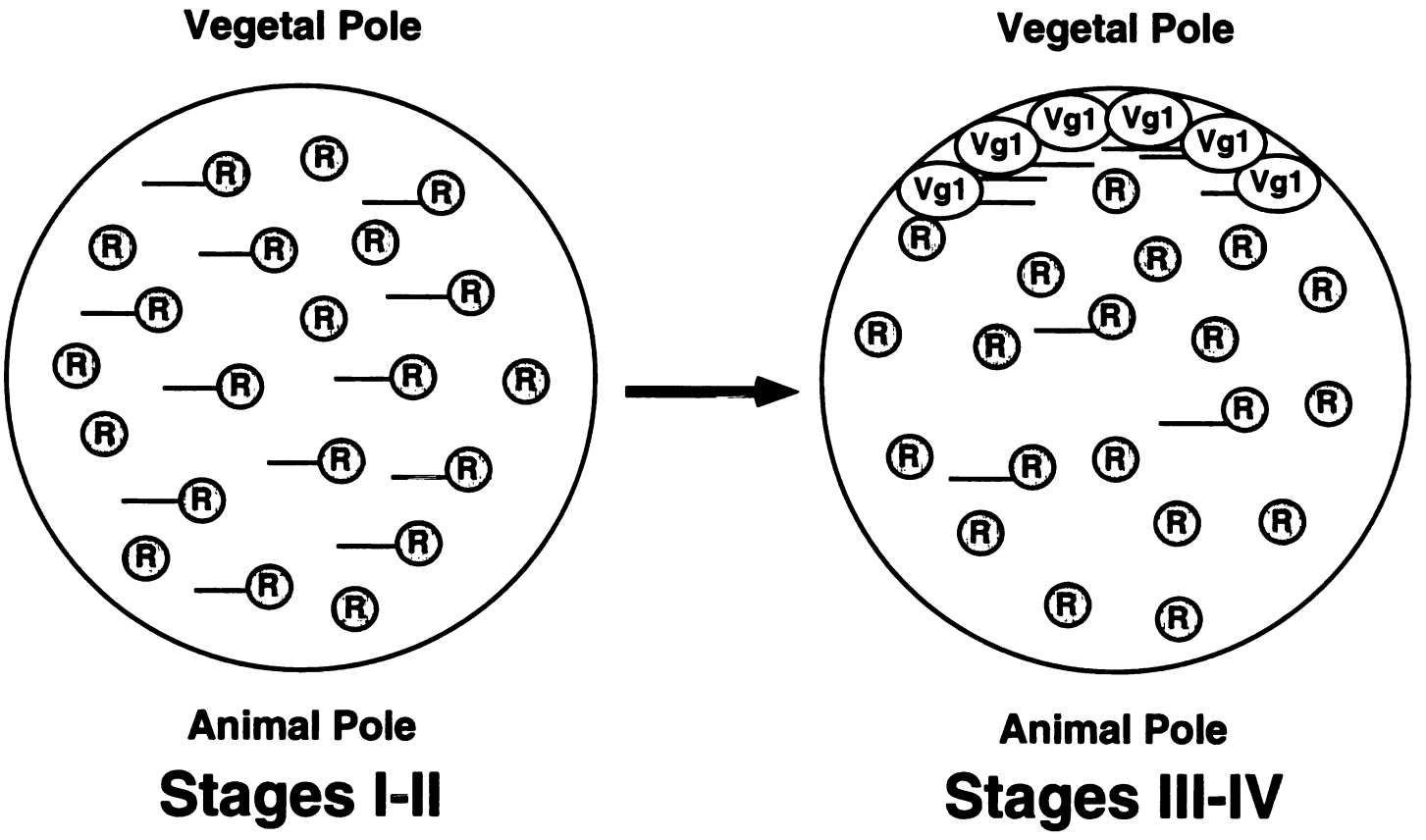

Vegetal Pole
Vegetal Pole
Vg1
Vg1
Vg1
Vg1
Vg1
Vg1
R
Animal Pole
Animal Pole
Stages I-II
Stages III-IV

Figure 6. Model for coordinate control of Vg1 mRNA localization and translation. During stages I-II of oogenesis, all Vg1 mRNA is complexed with the translational repression machinery (R) and is homogeneously distributed throughout the oocyte. During stages III-IV of oogenesis, Vg1 mRNA becomes localized to the vegetal pole where the repression machinery is displaced and Vg1 protein is synthesized. However, the translational repression machinery is still active throughout the oocyte during these stages and those Vg1 messages that have not been localized continue to be repressed.

Chapter 3

Abstract

Localization of *bicoid* (*bcd*) mRNA to the anterior and *oskar* (*osk*) mRNA to the posterior of the *Drosophila* oocyte is critical for embryonic patterning. Previous genetic studies implicated *exuperantia* (*exu*) in *bcd* mRNA localization, but its role in this process is not understood. Here, we have biochemically isolated Exu and show that it is part of a large RNAse sensitive complex that contains at least seven other proteins. One of these proteins was identified as the cold shock domain RNA binding protein Ypsilon Schactel (Yps), which we show binds directly to Exu and colocalizes with Exu in both the oocyte and nurse cells of the *Drosophila* egg chamber. Surprisingly, the Exu/Yps complex contains *osk* mRNA. This biochemical result led us to re-examine the role of Exu in the localization of *osk* mRNA. We discovered that *exu* null mutants are defective in *osk* mRNA localization in both nurse cells and the oocyte. Furthermore, both Exu/Yps particles and *osk* mRNA follow a similar temporal pattern of localization in which they transiently accumulate at the oocyte anterior and subsequently localize to the posterior pole. We propose that Exu is a core component of a large protein complex involved in localizing mRNAs both within nurse cells and the developing oocyte.

Introduction

Localization of mRNAs is used by many polarized cells as a means of restricting the distribution of a protein to a particular cytoplasmic domain (Bashirullah et al., 1998; Hazelrigg, 1998; St Johnston, 1995). While the types of localized transcripts vary, mRNA localization in all systems share several common features. First, the *cis*-acting mRNA localization elements generally reside within the 3′ untranslated region (3′ UTR) (Bashirullah et al., 1998; Kislauskis and Singer, 1992). Second, the transport of localized messages from the nucleus to their final destinations occurs along either actin filaments or microtubule tracks. Third, transcripts are anchored at their sites of localization through attachments to cytoskeletal elements and then activated for translation (Bassell and Singer, 1997; Gavis, 1997; Macdonald and Smibert, 1996; Oleynikov and Singer, 1998; Wilhelm and Vale, 1993). While these phenomena have been well documented, their molecular bases remain poorly understood.

One of the most extensively characterized systems for studying mRNA localization is the *Drosophila* oocyte. In the *Drosophila* egg chamber, an oocyte is linked to fifteen nurse cells by a network of cytoplasmic bridges called ring canals (Spradling, 1993). The nurse cells synthesize various mRNAs that are required for early embryogenesis, such as the *bcd* and *osk* transcripts, and transport them in a microtubule-dependent manner to discrete locations within the oocyte (Pokrywka and Stephenson, 1995; Pokrywka and Stephenson, 1991). *bcd* mRNA is localized to the anterior of

the oocyte (Berleth et al., 1988), and the resulting anterior-posterior gradient of the Bicoid homeodomain protein initiates a series of concentration-dependent transcriptional programs that establish the anterior pattern of the embryo (St Johnston and Nusslein-Volhard, 1992). In contrast, *osk* mRNA is transported from the nurse cells to the anterior of the oocyte, but is ultimately localized to the posterior of the oocyte where it becomes stably anchored (Ephrussi et al., 1991; Kim-Ha et al., 1991). The Oskar protein synthesized at this location recruits a number of additional components that are required for the formation of the abdomen and germ cells (Breitwieser et al., 1996; Ephrussi et al., 1991; Kobayashi et al., 1995; Smith et al., 1992).

Genetic screens have identified several mutants that have patterning defects due to the mislocalization of *bcd* and/or *osk* mRNAs. Mutations in some genes, such as *swallow* and *staufen*, cause only partial disruption of mRNA localization late in oogenesis (Berleth et al., 1988; St Johnston et al., 1991; St. Johnston et al., 1989; Stephenson et al., 1988). However, in *exuperantia* (*exu*) mutants, defects in *bcd* mRNA localization occur early in oogenesis and result in *bcd* mRNA being uniformly distributed in the mature oocyte (Berleth et al., 1988; St. Johnston et al., 1989). Time-lapse confocal microscopy has further shown that GFP-Exu forms particles that move in a microtubule-dependent manner and accumulate at the anterior and posterior of the oocyte (Theurkauf and Hazelrigg, 1998). Immunoelectron microscopy also has revealed that Exu is a component of large electron-dense structures called sponge bodies (Wilsch-Brauninger et

al., 1997). However, all of these previous studies have not determined whether Exu is associated with transported mRNAs or whether its role is more indirect, such as in delivering material required for anchoring mRNA to the oocyte anterior.

Various studies have shown that localized messages are organized into particles (Ainger et al., 1993; Bertrand et al., 1998; Ferrandon et al., 1994), suggesting that a large protein complex may be involved in recognizing, transporting, and anchoring localized messages. However, identifying the proteins associated with localized mRNAs has been a difficult undertaking. Previous biochemical studies uncovered proteins that specifically recognize the RNA localization sequences in the 3' untranslated region (3'UTR) of mRNAs (Deshler et al., 1998; Havin et al., 1998; Hoek et al., 1998; MacDonald et al., 1995; Ross et al., 1997). However, their roles in mRNA transport have not been confirmed by genetic analyses. An alternative strategy is to isolate the native ribonucleoprotein complexes involved in mRNA transport and then identify the associated proteins. To achieve this goal, we thought that the Exu protein might provide a useful biochemical handle for the purification of an mRNA transport particle. In this study, we demonstrate that Exu exists in a large RNAse-sensitive complex with at least seven other proteins, one of which is a cold shock domain RNA binding protein. Unexpectedly, *osk* mRNA is present in the Exu complex, and we have discovered that Exu is involved in posterior mRNA localization in addition to its previously described role in localizing mRNAs to the anterior. We

propose that Exu is part of a core complex that localizes both *osk* and *bcd* mRNAs within nurse cells and the developing oocyte.

Materials and Methods

Drosophila stocks

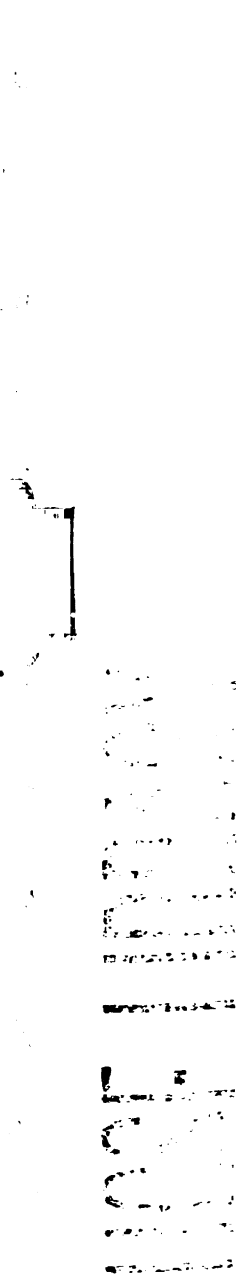
Oregon R (Ore) was the wild-type stock used for antibody staining and generation of wild type *Drosophila* extracts. Females from NG5/NG5; Sco/SM1, which contains an X-linked insertion of a P[CasNGE] *gfp-exu* transgene (Wang and Hazelrigg, 1994), were used for double labeling of Yps and Exu. For biochemical analysis, flies bearing a *gfp-exu-his*$_6$ transgene (16b-16) were used.

In order to construct the *gfp-exu-his*$_6$ transgene, the 5.5 kb SmaI- EcoRI *exu* genomic fragment was subcloned into pBlueScript II (SK) (Stratagene), to create pBS-*exu*5.5. pBS-*exu*5.5 was used as the template for *in vitro* mutagenesis to introduce BstEII and SphI restriction sites immediately upstream of exu's translation start codon (using the Bio-Rad Muta-Gene kit). Simultaneously, an 18 bp insertion encoding 6 histidine codons was introduced just upstream of *exu*'s stop codon. Correctly mutagenized plasmids were identified by restriction analysis for the presence of the BstEII and SphI sites, and DNA sequence analysis for the presence of the 18 bp his-encoding insertion. A 700 bp BstEII-SphI *gfp* fragment was excised from pEG1 (Wang and Hazelrigg, 1994), and subcloned into the BstEII and SphI sites of this mutagenized *exu* fragment. The resulting 6.2 kb *gfp-exu-his*$_6$ fragment was then excised by SmaI and EcoRI digestion, and inserted into pCasPeR4 through its StuI and EcoRI cloning sites, to create pWG16b. P-element transformation followed standard injection protocols (Spradling and Rubin,

1982), with transposase activity provided by the recipient strain (*y w ; Sb 2-3/TM6*). A transformant line bearing an insertion on the X chromosome was established (*w, 16b-16*), and genetic crosses with an exu^{SCO2}/CyO strain determined that the *gfp-exu-his*$_6$ transgene rescued the female sterility associated with the exu^{SCO2}mutation.

Extract Preparation

Extracts were prepared by flash freezing 50-60 ml of flies in liquid N_2. The frozen flies were ground to a fine powder with a pre-chilled mortar and pestle, with regular additions of liquid N_2 to keep the sample frozen. The fly powder was degassed for 5-10 min on ice in a 50 ml Falcon tube and mixed either 1:1 or 1:2 with *Drosophila* Extract Buffer (DXB: 25 mM HEPES, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose) containing 10 µg/ml aprotinin, leupeptin, pepstatin, and 1 mM PMSF. The extracts were homogenized with 10 strokes of the B dounce in a 50 ml Dounce homogenizer, followed by 10 strokes of the A dounce, and then centrifuged at 10,000xg, 15 min at 4°C in the Beckman TLX ultracentrifuge. The supernatant was collected and centrifuged a second time. The supernatant from the second spin was collected, aliquoted, frozen in liquid N_2, and stored at -80°C. Hand dissected extracts were prepared by dissecting 50 ovaries into 0.7 ml of modified *Drosophila* Extract Buffer (mDXB: 25 mM HEPES, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 125 mM sucrose) containing 10 µg/ml aprotinin, pepstatin, leupeptin, and 1 mM PMSF. The sample was then homogenized with 15 strokes in a 2 ml Dounce homogenizer (Wheaton,



Inc.) at 4°C. The extract was centrifuged at 10,000xg, 10 min at 4 °C to collect the supernatant.

Sucrose Density Gradient Analysis of Exu Complex

Extract from hand dissected ovaries (250 µl) was loaded on a 5 ml 5-40% sucrose gradient made with DXB containing 10 µg/ml leupeptin, pepstatin, and aprotinin. For frozen *Drosophila* extracts, 150 µl of ~10 mg/ml extract was diluted 1:1 with sucrose-free DXB containing 10 µg/ml pepstatin, leupeptin, aprotinin, and 1 mM PMSF. The diluted extract was centrifuged at 10,000xg for 5 min in a microcentrifuge and the supernatant loaded on a 5 ml 5–40% sucrose gradient. The gradients were centrifuged at 237,000xg for 4 hr in a Beckman SW-55 rotor. 300 µl fractions were collected from the gradient and precipitated with 1/10 volume TCA using 20 µg aprotinin as a carrier. The precipitate was resuspended in 30 µl sample buffer and 15 µl was loaded onto SDS-PAGE gel for immunoblot analysis. For RNAse shift experiments, the extract (250 µl) was either treated with 2.5 µg of RNAse A for 10 min at 4°C, followed by addition of 2000U of RQ1 RNAsin (Promega) or treated for 10 min at 4°C with 2.5 µg of RNAse A that had been preincubated with 2000U of RQ1 RNAsin. These samples were loaded onto a 5 ml 10-40% sucrose gradient and centrifuged as above.

Two Step Purification of GFP-Exu-His_6

Five ml of ~10 mg/ml *Drosophila* extract (made with 5 mM β-mercaptoethanol instead of 1 mM DTT) were incubated with 0.5 ml of Ni-NTA resin in the presence of 0.1% Triton X-100 for 1 hr at 4°C. The resin was washed once in batch with 10 ml of DXB150 (DXB with 150 mM KCl) supplemented with 0.1% Triton X-100 (Wash 1) followed by a 10 ml column wash (Wash 2). The column was eluted with 250 mM imidazole in DXB150, and 0.5 ml fractions collected. The peak fractions were pooled and immunoprecipitated as described.

Antibody Generation

The full length coding region and the first 160 amino acids of Yps (Yps160) were each cloned into pGEX-2T and expressed as C-terminal fusions to glutathione S-transferase (GST) in *E. coli*. Soluble GST-Yps and GST-Yps160 were purified on a glutathione affinity column, eluted with glutathione, and injected into rabbits (antiserum production by BABCO, Berkeley, CA). GST-Yps did not express as a full length protein in E. coli, but rather yielded a protein product ~10 kDa larger than GST-Yps160. Antisera to GST-Yps160 was affinity purified by preabsorption on an Affi-Gel column coupled with GST, followed by affinity purification with an Affi-Gel column coupled with GST-Yps160. This affinity purified antibody was used for all experiments except for Figure 8C,D where the GST-Yps antisera was used. The antisera against GST-Yps gave identical results to the affinity purified α-Yps160 antibody in immunofluorescence experiments.

GFP was expressed in *E. coli* and purified on a Q-sepharose column followed by a phenyl sepharose column. The purified GFP was injected into rabbits for antisera production (BABCO, Berkeley, CA). Antisera to GFP was affinity purified with an Affi-Gel column coupled with GFP. This antibody recognizes GFP in both immunoblots and immunoprecipitations.

Antisera against a peptide (NRRGGRQSVKDARPSSC; a.a. 422-437) from Exu were generated (QCB, Inc.) and affinity purified with peptide coupled to Sulfolink gel (Pierce, Inc.). This affinity purified antibody recognizes Exuperantia in both immunoblots and immunoprecipitations.

Immunoblots

For immunoblot analysis, samples were run on SDS-polyacrylamide gels and then transferred to nitrocellulose by semi-dry transfer. The membrane was blocked in TBS (TBS: 20 mM Tris-Cl, pH 7.5, 200 mM NaCl), 0.1% Tween-20, 10% nonfat dry milk and then incubated with either 1:2000 dilution of α-Exu rabbit antibody (gift of Paul MacDonald) or 5 μg/ml of affinity purified α-Yps rabbit antibody in TBS, 0.1% Tween-20, 5% BSA. Protein was detected by chemiluminescence using horseradish peroxidase conjugated donkey α-rabbit Ig (Amersham) diluted 1:2500 in TBS containing 0.1% Tween-20 and 5% BSA.

Immunoprecipitations of Yps and GFP

125 μl of Protein A agarose beads (Gibco/BRL) were washed 6 times with PBS (PBS: 137 mM NaCl, 2.6 mM KCl, 10 mM $NaHPO_4$, 1.7 mM KH_2PO_4) + 0.1%

Triton X-100 (PBST). 50 µg of antibody were added to the beads in a final volume of 300 µl and mixed for 30 min at room temperature. The beads were washed once with PBST followed by two washes with 0.2 M sodium borate, pH 9.0. Dimethyl pimelidate was then added to a final concentration of 20 mM and the sample mixed for 1 hr at room temperature. The beads were washed three times with 0.2 M ethanolamine, pH 8.0 and mixed 2 hr at room temperature. The beads were pre-eluted with three 1 ml washes of 100 mM glycine, pH 2.5 followed by three washes with DXB.

Fresh 10 µg/ml leupeptin, pepstatin, and 1 mM PMSF were added to 1 ml of frozen *Drosophila* extract (~10 mg/ml) and centrifuged at 10,000xg for 5 min in a microcentrifuge. 850 µl of supernatant were immunoprecipitated with 50 µl of antibody-coated beads for 1 hr at 4°C with gentle shaking. The beads were washed four times with DXB containing 10 µg/ml leupeptin, pepstatin, and 1 mM PMSF and then eluted with two 150 µl washes and one 200 µl wash of 100 mM glycine, pH 2.5. The elutions were neutralized with 200 µl of 0.5 M HEPES, pH 7.6 and any residual beads removed by centrifuging at 10,000xg for 5 min in a microcentrifuge. The supernatant was precipitated with 1/10 volume TCA in the presence of 20 µg aprotinin as a carrier. The sample was resuspended in sample buffer for further analysis. When RNAse-sensitivity of the immunoprecipitation was assayed, the incubation of extract with antibody-coated beads was performed in the

presence of 300 µg of RNAse A. Immunoprecipitations of the Exu complex from sucrose gradients were performed on fractions pooled from five 5-40% sucrose gradients loaded with frozen *Drosophila* extract using our standard conditions (see above).

Microsequence Analysis of p57/Yps

p57 was immunoprecipitated as described, separated by SDS-PAGE, and then stained with Coomassie Blue. After removing the protein band, an in-gel digestion with Endoproteinase Lys-C (Roche Diagnostics, Indianapolis, IN) was carried out as described (Hellman et al., 1995). Gel-extracted peptides were then fractionated using a Vydac microbore C8 column (The Separations Group, Hesperia, CA), and individual peptides subjected to Edman degradation with a Protein Sequencer, Model 492 (PerkinElmer Biosystems, Foster City, CA). BLAST searches with these peptides identified p57 as Yps. Six ESTs corresponding to *yps* (GM14045, LD01826, GM03816, LD18388, GM02535, LD01538) were obtained (BDGP/HHMI EST Project) and sequenced.

Immunoprecipitations for RT-PCR

Immunoprecipitations were carried out as above, but the antibody was not crosslinked to the protein A agarose beads (Gibco/BRL). Beads were washed four times DXB200 (DXB, with 200 mM KCl) containing 10 µg/ml leupeptin, pepstatin, and 1 mM PMSF. Beads were then resuspended in 200 µl buffer (100 mM HEPES, pH 6.8, 150 mM NaCl, 12.5 mM EDTA, 1% SDS) and heated to 65°C for 10 min. The beads were pelleted and the supernatant from two immunoprecipitations was phenol/chloroform extracted followed by an

ethanol precipitation using 20 µg glycogen as carrier. The pellet was resuspended in 50 mM HEPES, pH 6.8, 1 mM $MgCl_2$ and treated with 10 U RQ1 DNAse I (Promega) for 30 min at 37 °C. The sample was then phenol/chloroform extracted, and ethanol precipitated a second time. The RNA sample was reverse transcribed (RT) using the Superscript preamplification system (Gibco/BRL) and 2 µl of 10, 100, 1000 fold dilutions of the RT product were amplified in a 50 µl PCR reaction (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTPs, 2.5 U Taq polymerase, 15 pmol primer). Cycling conditions were 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 55°C, and then 1 min at 72°C. Primers were tested by using the same RT-PCR conditions as above except 5 µg of total RNA from extracts was used in the RT reaction and only 30 cycles of PCR were performed. The primers used were: bcd2761: 5'-gtcggatcctgggtcgaccaatgtcaatggcg-3', bcd3738: 5'-gtcgaattcgctcttgtccagacccttcaaagg-3', osk860: 5'-gtcggatccaatggaatcacgatatcgagcatca-3', osk1630: 5'-gtcgaattcttacgctggcttgctggtagaaat-3', nos1110: 5'-gtcggatccgatccttgaaaatctttgcgcaggt-3', nos2221: 5'-gtcgaattctcgttgttattctcacaaaagacgca-3', pgk1800: 5'-gtcggatccgccaagaagaataacgtgcagttgc-3', pgk2280: 5'-gtcgaattccgctggtcaatgcacgcacgc-3'. RT-PCR primers were designed for *osk*, *nos*, and *bcd* to span an intron to easily separate RT-PCR products from products

that were amplified due to genomic contamination. Products for *osk*, *nos*, and, *bcd* were subcloned and their identity confirmed by DNA sequencing.

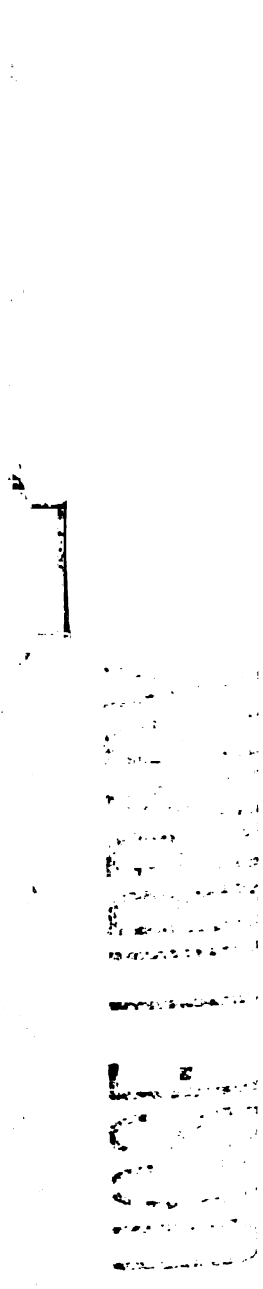
Immunofluorescence

0-1 day old females were collected and fed 1-2 days with dried bakers yeast, in the presence of males. Ovaries were dissected into PBT (PBS + 0.1% Triton X-100) and fixed in 600 µl of heptane and 100 µl of fixative (6% formaldehyde, 16.7 mM KPO_4 pH 6.8, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$), rocking at room temperature for 9 min. The ovaries were then washed several times in PBT, for a total of 15 min. Ovaries were permeablized in PBS + 1% Triton X-100 for 5 hr, blocked in 0.5% BSA in PBT for 30 min, and incubated in a 1:500 dilution of the primary antibody in PBT overnight at 4°C. The secondary antibody (rhodamine-conjugated goat α–rabbit Fab fragments; Jackson Immunochemical Research, Inc) was simultaneously preabsorbed to fixed ovaries at a dilution of 1:500 in PBT, overnight at 4°C. After removal of the primary antibody, the ovaries were washed in PBT (5 x 5 min) at room temperature, and incubated in the preabsorbed secondary antibody for 3 hr at room temperature. The ovaries were then washed in PBT (5 x 5 min), and either mounted in 40 µl Fluoromount-G (Southern Biotechnology Associates, Inc) or incubated overnight in 60% glycerol in PBT at 4°C, then mounted in 40 µl of 60% glycerol. Imaging was performed on a Biorad MRC600 laser confocal unit attached to a Zeiss Axioplan microscope.

Binding Assays for *In Vitro* Translated Exu and Yps

Site directed mutagenesis and PCR were used to create unique restriction sites in order to attach N-terminal epitope tags in-frame to *exu* and *yps* cDNAs. DNA encoding the 3x myc tag was obtained from T7myc-Gsp1wt vector (gift of Erin O'Shea) and joined to the 5' end of *yps*. DNA encoding the 3x Hemagglutinin (HA) tag was obtained from the pGTEP vector (gift of Joachim Li) and joined to the 5' end of *exu*. Each of the two fusions were then subcloned into the Nco I and Eco RI sites of the pSPBPI transcription vector (gift of Vishu Lingappa); this vector was derived from pSP64 and includes a 5'UTR and strong Kozak consensus region from *Xenopus* globin cDNA.

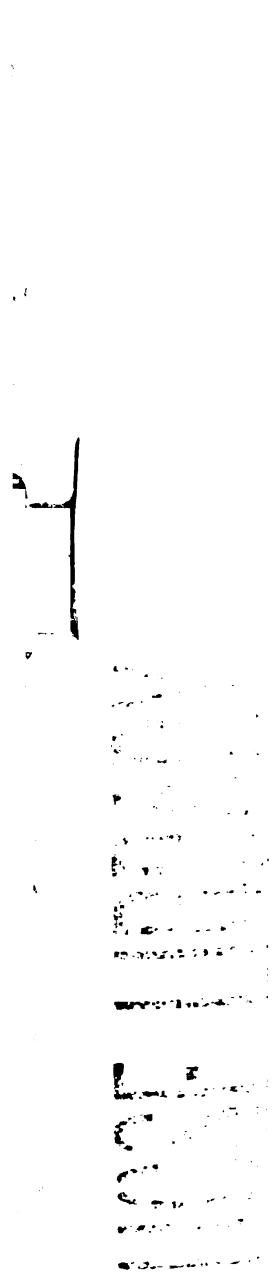
A coupled transcription/translation system (Promega) was used to *in vitro* translate tagged Exu and Yps. 600 ng of plasmid DNA was added to a 50 µl transcription/translation mix. The myc-Yps transcription/translation mix contained ^{35}S-Methionine (Translabel; ICN) while the HA-Exu mix did not. After a 90 min incubation at 30°C, 0.5 µl of 400 nM 7-methylguanosine 5-monophosphate (CAP Analog) and 5 µl of 10 mg/ml RNAse A were added to each reaction. 5 µl of radiolabelled myc-Yps was either incubated alone or mixed with cold HA-Exu and coincubated for 30 min at room temperature. Afterwards, 490 µl of DXBT250 (DXB containing 250 mM KCl and 0.1% Triton X-100) was added to the coincubation mixes prior to the immunoprecipitations.



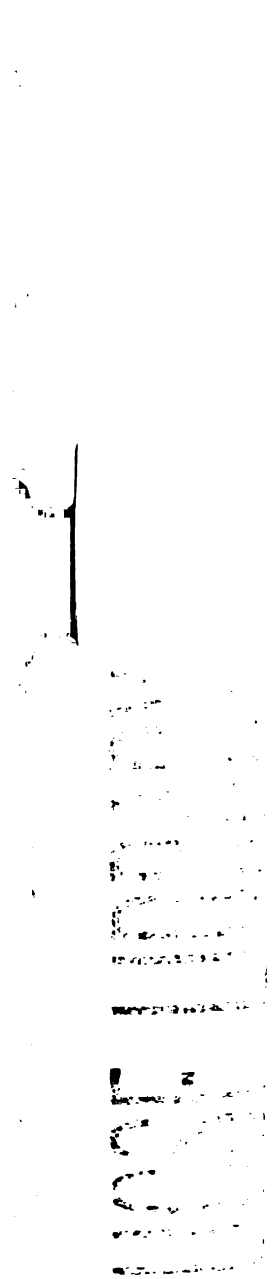
For immunoprecipitations, 0.5 µl of α-HA (16S12 monoclonal; gift of Dave Morgan) was added to translations and set on a rotator at 4°C for 60 min. A 30 µl slurry containing 10 µl of Protein A beads (Life Technologies, Inc) was added to the translation/antibody mix and incubated for another 60 min at 4°C on a rotator. The beads were then pelleted in a low speed microfuge for 2 min and the supernatant was removed. After washing with DXBT250 (3 x 1 ml), the beads were resuspended in SDS sample buffer and boiled for 5 min. 7.5 µl were loaded on 4-12% SDS-PAGE gel and processed for autoradiography.

oskar *In Situ* Hybridization

Whole mount *in situ* hybridization to ovaries was performed as previously described (Tautz and Pfeifle, 1989) with several modifications. Ovaries from well fed, 2-3 day old females raised at 18°C (Oregon R or exu^{SC02}/Df(2)MK1) were dissected into PBT and fixed 15 minutes in 8:1:1 (4% paraformaldehyde in PBT: Clorox bleach: dimethyl sulfoxide). The ovaries were washed 2 x 5 min in PBT, and 5 min each in 3:1, 1:1 and 1:3 PBT: methanol, then 2 x 5 min in methanol, and stored at -20°C. Prior to hybridization, the ovaries were rehydrated into PBT by 5 min washes in 3:1, 1:1, 1:3 methanol: PBT and 2 x 5 min in PBT. After teasing apart the ovarioles slightly, the ovaries were treated 15 min with 50 µg/ml proteinase K (Boehringer Mannheim) in PBT, washed twice quickly in PBT, and postfixed 10 min in 4% paraformaldehyde in PBT. Following 5 x 5 min



washes in PBT, the ovaries were equilibrated into hybridization buffer (HB: 5xSSC, 0.1% Tween 20, 50 μg/ml heparin, 0.1 mg/ml denatured salmon sperm DNA, 50% formamide) by 5 min incubations in 3:1, 1:1, and 1:3 PBT: HB, followed by a one hr incubation at 45°C in HB. Hybridization was done in 10 μL of probe in HB (see below), overnight, at 45°C. Excess probe was removed by three 20 min washes in HB, followed by 10 min washes in 3:1, 1:1, and 1:3 HB:PBT, all at 45°C. The ovaries were then washed 5 x 5 min in PBT at room temperature, then incubated in an alkaline phosphatase-conjugated anti-digoxygenin antibody (Boehringer Mannheim) at a dilution of 1:2000 in PBT for 1 hr. Prior to incubation, the antibody was preabsorbed to fixed ovaries for at least one hr. The ovaries were washed 5 x 5 min in PBT and 2 x 5 min in alkaline phosphatase (AP) staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5, 0.1% Tween 20). For antibody detection, 1 ml AP staining buffer, 4.5 μl NBT and 3.5 μl X-Phosphate (Boehringer Mannheim) were added to the ovaries, and incubated 3-10 min. We found that a short staining time was crucial in order to prevent saturation of the color reaction, which masked differences between the genotypes. The ovaries were then washed several times in PBT for a total of 45 min and equilibrated overnight at 4°C in 60% glycerol. Ovaries were mounted in 60% glycerol, and visualized on a Nikon Eclipse E800 microscope with a Nikon Plan Fluor 20x objective, under Nomarski optics. Photographs were taken with a Nikon FX-35WA camera on Kodak Elite Chrome slide film, and scanned into Adobe Photoshop with a Polaroid SprintScan 35 slide scanner.



Probes were prepared by labeling a plasmid containing the *osk* cDNA, PNBosk7 (courtesy of R. Lehmann) with dig-dUTP using DIG-Nick Translation Mix (Boehringer Mannheim). After ethanol precipitation, the probe was resuspended in 100 μl HB. Just prior to use, the probe was denatured by boiling for 5 min, and cooled on ice.

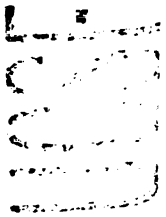
Results

Exuperantia (Exu) exists in a large RNAse sensitive complex

In order to define the biochemical properties of Exu, we determined its size in *Drosophila* extracts using sucrose density gradients. In extracts made from either hand dissected ovaries (Figure 1A) or whole flies that were frozen in liquid N_2 (not shown), Exu migrated in a very broad distribution with a significant fraction of Exu migrating larger than 20S (Figure 1A). In contrast, *in vitro* translated Exu sedimented at 4.5S, the value expected of a monomeric 58 kDa protein (data not shown). Therefore, the large size of Exu in *Drosophila* extracts must be due to its association with additional proteins and/or RNAs. To test whether the Exu complex contains RNA, the extract was treated with RNAse A prior to sedimentation analysis. This treatment caused the complex to migrate as a 7S species; this shift was not due to proteolysis, since a control reaction in which RNAse A was pre-mixed with RNAse inhibitor did not affect Exu migration (Figure 1B). These results demonstrate that Exu is associated with a large RNA containing complex.

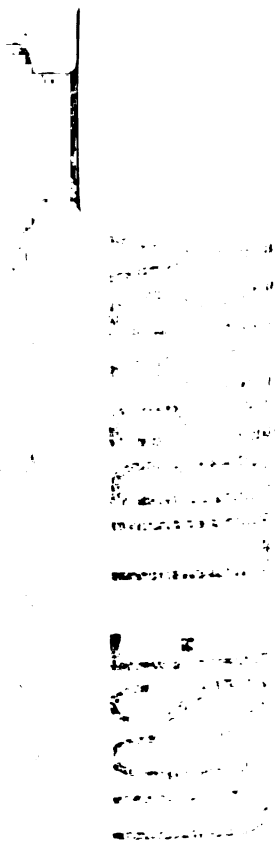
The RNA binding protein, Ypsilon Schactel (Yps), copurifies with Exu

In order to identify the protein components of the Exu complex, GFP-Exu was immunoprecipitated from whole fly extracts prepared from a GFP-Exu expressing fly line using an α-GFP antibody. The GFP tag does not impair Exu protein function, since the *gfp-exu* transgene fully complements a null allele of *exu* (*exu*^{SCO2})(Wang and Hazelrigg, 1994). Figure 2 shows that



seven polypeptides of 57, 74, 76, 78, 82, 88, and 147 kDa coimmunoprecipitated specifically with GFP-Exu, but not with control IgG-coated beads. A similar set of polypeptides coimmunoprecipitated with GFP-Exu from an extract made from hand-dissected ovaries, indicating that this complex is present within the female germ line (data not shown). This same set of polypeptides also coimmunoprecipitated with Exu from wild type fly extracts using an antibody directed against a COOH-terminal peptide of Exu (data not shown; see Experimental Procedures). Of the coimmunoprecipitated proteins, the 57, 74, 76, 78, and 82 kDa proteins were present in amounts comparable to that of GFP-Exu, while the 88 kDa and 147 kDa proteins were clearly substoichiometric. When the extract was extensively treated with RNAse A, only the 57 kDa protein remained associated with GFP-Exu (Figure 2). Taken together with our gradient analysis (Figure 1B), these results suggest that Exu and p57 are components of a 7S RNAse-resistant core complex; the other polypeptides (p74, p76, p78, p82, p88, and p147) all require the presence of RNA in order to associate with Exu.

To confirm that the 57 kDa polypeptide was a bona fide Exu-associated protein, we used a different purification strategy to isolate Exu complexes. Using flies that express Exu with an amino-terminal GFP tag and a carboxy-terminal His_6 tag, extracts were subjected to a two-step purification consisting of binding to a Ni-NTA column, elution with imidazole, and then immunoprecipitation with the α-GFP antibody (Figure 3). The 57 kDa protein consistently co-purified stoichiometrically with GFP-Exu-His_6



through this two step affinity purification, confirming that it is a true Exu-associated polypeptide. The other polypeptides in the 74-82 kDa range were identified in some of our preparations, but their presence and amount was highly variable, possibly due to RNA degradation during the procedure or instability of the complex during imidazole elution from the column.

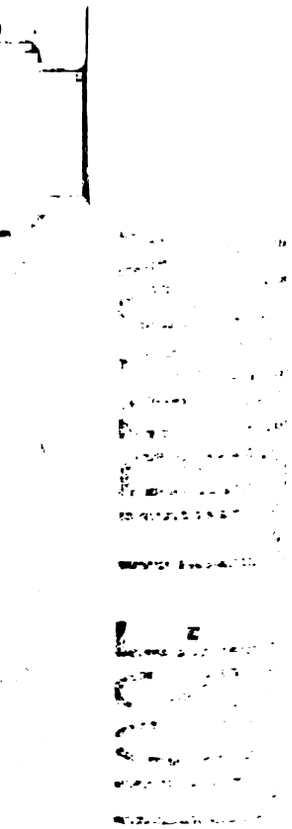
To identify the 57 kDa Exu-associated protein, we microsequenced three tryptic peptides from the purified protein. The sequence from two of the three peptides matched a previously identified protein, the product of the *ypsilon schactel* (*yps*) gene (Figure 4A). Yps is a member of the cold shock family of RNA binding domain proteins and was identified as part of a degenerate PCR screen to identify cold shock domain containing genes from *Drosophila melanogaster* (Thieringer et al., 1997). However, the third peptide only matched the Yps sequence in a reading frame other than the published open reading frame. In order to rule out the possibility that *yps* expression is subject to ribosomal frameshifting or RNA editing, we obtained and sequenced 6 independent *yps* ESTs (Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished). Our sequence revealed that the original *yps* sequence contained several sequencing errors and that the correct open reading frame contains all three microsequenced peptides (Figure 4A). The cold shock domain of Yps shows extensive sequence identity to other cold shock domain proteins (Figure 4B). This domain has been shown in several studies to bind RNA, although its ability to recognize specific substrates remains uncertain (Bouvet et al., 1995; Matsumoto et al.,

1996; Murray, 1994). Beyond the cold shock domain, Yps exhibited no significant homology to any other protein except YB-1, a cold shock domain protein from *Drosophila silvesteris* (AAC06034). Since the YB-1 protein is 70% identical to Yps across the entire length of the protein, it is likely to be a true ortholog of Yps. No function was assigned to either YB-1 or Yps in these prior studies.

Yps is a component of the Exu complex

To further characterize Yps, we prepared affinity-purified antibodies against bacterially-expressed Yps (a.a. 1-160). These antibodies recognized the 57 kDa Yps protein in crude extracts by immunoblot (Figure 5A). Using the Yps antibody, we found that Yps comigrates with Exu in sucrose gradients and is distributed broadly in the 20-60S size range (Figure 5B). In order to rule out the possibility that Exu and Yps are components of distinct complexes of similar size, GFP-Exu was immunoprecipitated from individual gradient fractions and immunoblotted with the Yps antibody (Figure 5C). This experiment showed that Exu and Yps coimmunoprecipitate together across the gradient, arguing strongly that Exu and Yps are part of the same complex.

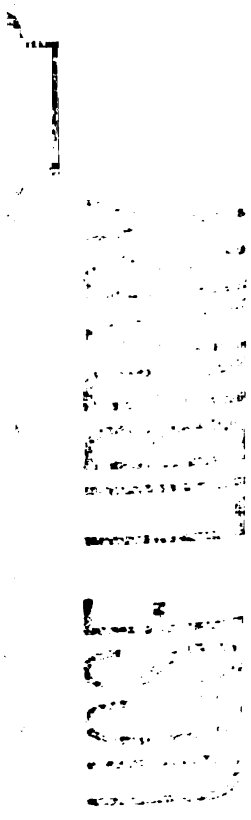
To provide further evidence for an Exu-Yps complex, we immunoprecipitated GFP-Exu extracts with our α-Yps polyclonal antibody. In agreement with our previous immunoprecipitation results, immunoblots showed that GFP-Exu specifically coimmunoprecipitates with Yps (Figure 5D). However, immunoblots showed only a weak GFP-Exu band in the Yps



immunoprecipitate. The inefficient coimmunoprecipitation of GFP-Exu with Yps is probably due to the fact that our α-Yps antibody may displace Exu from the complex, since it was raised against the Exu binding region of Yps (discussed below; Figure 6). The Yps immunoprecipitates also contained the same six proteins (p74, p76, p78, p82, p88, and p147) that strongly coimmunoprecipitated with GFP-Exu and were present in similar stoichiometries (Figure 5E). The coimmunoprecipitation of these six proteins with Yps was diminished by RNAse treatment, as was observed in our previous Exu immunoprecipitation experiments (Figure 5E). The ability of Yps and Exu antibodies to coimmunoprecipitate the same set of polypeptides argues that these proteins are bona fide components of the Exu-Yps complex.

Exuperantia binds directly to the amino terminal region of Yps in the absence of RNA or other proteins

The coimmunoprecipitation of Exu and Yps after RNAse A treatment suggested, but did not prove, that Exu and Yps bound directly to each other. To test this idea, we examined the Exu-Yps interaction in an *in vitro* translation reaction (Figure 6). Myc-tagged Yps was *in vitro* translated in the presence of ^{35}S-methionine and then added to an unlabelled *in vitro* translation of HA-tagged Exu. Prior to mixing, each translation reaction was treated with RNAse A to eliminate any residual RNA from the translation reaction. When HA-Exu was immunoprecipitated from the combined mixture with the α-HA antibody, the ^{35}S-labelled myc-Yps protein was coimmunoprecipitated. The amount of myc-Yps that coimmunoprecipitated



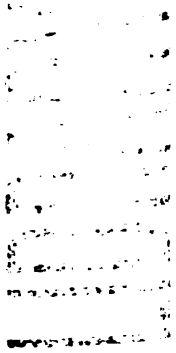
with HA-Exu was approximately half of the amount of myc-Yps that was immunoprecipitated directly with the α-myc antibody, showing that Yps is predominantly bound to Exu under these experimental conditions. These results demonstrate that the additional RNA and protein components of the native Exu complex are not required for Exu and Yps to associate stably with each other (Figure 6).

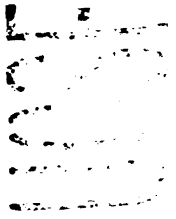
In order to determine which region of Yps is important for binding to Exu, deletions of myc-Yps were assayed for their ability to bind HA-Exu *in vitro*. The amino terminal region (1-160 a.a.) of Yps, which contains the cold shock domain, bound to Exu at the same efficiency as the full length protein (Figure 6). However, the minimal cold shock domain (56-151 a.a.) did not bind to Exu in this assay (Figure 6), suggesting that the sequences flanking the cold shock domain are likely to contribute to the Exu binding site. The proline-rich carboxy terminus of Yps presumably is not sufficient for binding. However, this could not be assessed experimentally, since this region did not stably express either *in vitro* or in bacteria.

osk mRNA is present in the Exu-Yps complex

The shift in the size of the Exu/Yps complex after RNAse treatment suggested that it might be directly involved in mRNA localization. To test this idea, we immunoprecipitated either Exu or Yps from extracts and analyzed the pellet for the presence of various localized (*bcd*, *osk*, *nanos* (*nos*)) messages and a housekeeping message (*phosphoglycerokinase* (*pgk*)). We analyzed these four transcripts, because each gene appears in the EST

database (Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished) at roughly equivalent frequencies indicating that these messages are likely to be present in comparable amounts. Furthermore, *bcd*, *osk*, and *nos* are localized differently during oogenesis. *bcd* mRNA is localized to the anterior of the oocyte beginning at stage 7 of oogenesis; *osk* mRNA is localized transiently to the anterior of the oocyte during stages 8/9 and is exclusively localized to the posterior by the end of stage 9. *nos* mRNA is localized to the posterior of the oocyte during late oogenesis. Using a reverse transcription-polymerase chain reaction (RT-PCR) assay, we amplified *bcd*, *osk*, *nos*, and *pgk* transcripts from total RNA from extracts (Figure 7A). When GFP-Exu immunoprecipitates were analyzed by RT-PCR, *osk* transcript (Figure 7B), but none of the others (data not shown), was amplified by RT-PCR. The identical result was obtained when RT-PCR was performed on immunoprecipitations with the α-Yps antibody, while control IgG immunoprecipitations yielded no *osk* RT-PCR signal (Figure 7B). In order to rule out artifacts due to signal saturation, we serially diluted the RT product from our immunoprecipitations prior to conducting the PCR. *osk* mRNA was consistently detected in the 10, 100, and 1000 fold dilutions of the RT product, and the signal decreased with increasing dilution (Figure 7B). This result indicates that the PCR reaction is approximately in the linear range and that signal saturation is not a factor in our assay. Therefore, the RT-PCR assay demonstrates that both Yps and Exu are associated with a complex that contains *osk* mRNA. This was an unanticipated result, since

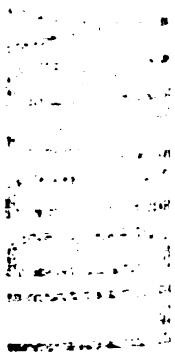


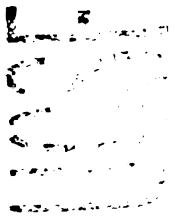


previous genetic studies only reported a role for *exu* in the localization of *bcd* mRNA to the anterior of the oocyte. We cannot rule out that the Exu-Yps complex contains *bcd* mRNA, since our negative result could reflect technical difficulties, such as poor *bcd* mRNA stability. However, our RT-PCR results suggested the unanticipated possibility that *exu* is involved in posterior mRNA localization.

Yps co-localizes with Exu in oocytes and nurse cells and accumulates at the posterior pole during mid-oogenesis

In order to learn more about the *in vivo* role of Yps in RNA localization, *Drosophila* ovaries were labeled with affinity-purified antibodies to the amino terminus (1-160 a.a.) of Yps. Immunofluorescence staining revealed a strong Yps signal in both the germ cells and follicle cells of developing egg chambers (Fig. 8). In contrast, labeling with secondary antibody alone produced a much weaker background signal (not shown). This result, together with the specificity of our α-Yps antibody by immunoblot (Figure 5A), argues that the staining we observe is specific for Yps. Examination of different stage egg chambers (see Spradling, 1993, for staging) revealed that Yps accumulates in the oocyte during stages 1-7. This signal was stronger at the posterior of the oocyte in early stages, although it was also present throughout much of the oocyte cytoplasm (Figure 8A). In both early and mid-stage egg chambers, Yps exhibited a particulate staining that was frequently concentrated around the nurse cell nuclei (Figure 8B). At stages 8 and 9, faint anterior localization was sometimes apparent in the





oocyte (Figure 8C), and during stages 9 and 10, Yps accumulated at the posterior of the oocyte (Figure 8C, D).

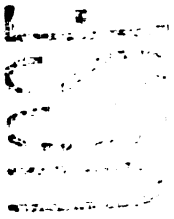
The localization of Yps during oogenesis was very similar to the previously observed distribution of GFP-Exu (Wang and Hazelrigg, 1994). In order to compare the distributions of Exu and Yps directly, we immunostained Yps in egg chambers expressing GFP-Exu. Individual particles containing both proteins were detected in the nurse cells (Figure 8E,F). The early accumulation of Yps in the oocyte (stages 1-7), its accumulation at the oocyte anterior (stages 8-9), and its later localization to the posterior pole (stages 9-10) all coincide with the localization of Exu protein and *osk* mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991; Wang and Hazelrigg, 1994). The agreement between our biochemical and *in vivo* localization studies further support a role for the Exu-Yps complex in the localization of *osk* mRNA. This hypothesis was tested directly by genetic studies, as described below.

exu mutants have defects in _osk_ mRNA localization

Whole mount *in situ* hybridization was performed on ovaries from wild type (Oregon R) and *exu* null (exu^{SCO2}/Df(2R)MK1) females to determine whether *exu* is required for any aspect of *osk* mRNA localization. In ovaries from wild type females, *osk* mRNA is often concentrated in apical patches in the nurse cells of stage 9 and 10 egg chambers (Pokrywka and Stephenson, 1995)(Figure 9A). In contrast, *osk* mRNA was dispersed in the nurse cells of *exu* egg chambers of the same stages (Figure 9B). Thus, *exu* is required for

osk mRNA to be correctly localized within nurse cells. Interestingly, *bcd* mRNA is also localized to apical patches in the nurse cells in an *exu*-dependent manner (St. Johnston et al., 1989). While the functional significance of this nurse cell localization is unknown, the fact that *exu* mutants disrupt this localization for both *osk* and *bcd* mRNAs indicates that at least some of the components required for this localization are common to both transcripts.

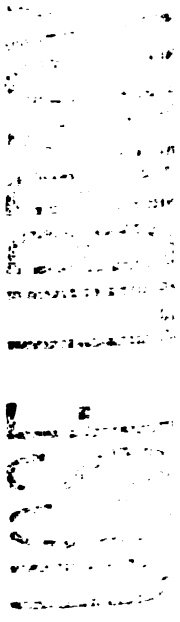
exu mutants also caused a partial defect in *osk* mRNA localization to the posterior pole. In ovaries from wild type females, a strong *osk* mRNA signal was detected at the posterior pole in 90% (n=90) of wild-type stage 9 and stage 10 oocytes (Figure 9C); the remainder (10%) showed reduced (9%) or barely detectable (1%) posterior *osk* mRNA signal. In contrast, only 64% of stage 9 and stage 10 egg chambers from *exu* mutants (n=210) showed a strong *osk* mRNA signal at the posterior of the oocyte, and this signal was consistently weaker than that observed in the wild-type egg chambers; the remainder (36%) showed reduced (23%), barely detectable (8%) (Figure 9D), or no (5%) posterior *osk* mRNA signal. Thus, mutations in *exu* cause a defect in the amount of *osk* mRNA that is localized to the posterior pole during stages 9 and 10 of oogenesis.



Discussion

While mRNA localization in *Drosophila* has been the subject of extensive genetic analysis, only a few attempts have been made to characterize biochemically the proteins associated with localized messages. In this study, we have demonstrated that Exuperantia, a protein shown by genetic studies to be involved in mRNA localization, is part of a large RNAse sensitive complex. Through a combination of affinity chromatography and immunoprecipitation experiments, we have uncovered seven proteins associated with the Exu complex, one of which is a new RNA binding protein, Ypsilon Schactel (Yps).

To our knowledge, this is the first isolation of a native RNP complex involved in RNA localization. There are several pieces of evidence that argue against the Exu-containing complex being a biochemical artifact. First, the complex was identified using three different antibodies for immunoprecipitation. Second, the complex that we isolated from total fly homogenates is not an artifact of mixing proteins from different tissues, since it is also present in extracts made from hand-dissected ovaries. Third, Exu and Yps also copurified after metal affinity chromatography, and the two proteins interact *in vitro* in the absence of RNA. Fourth, the Exu and Yps colocalize by immunofluorescence indicating that they form a complex *in vivo*. Fifth, Exu, Yps and *osk* mRNA all show a transitory accumulation at the anterior in mid-oogenesis and then colocalize at the posterior pole. Finally, the relevance of the biochemical association with *osk* mRNA is

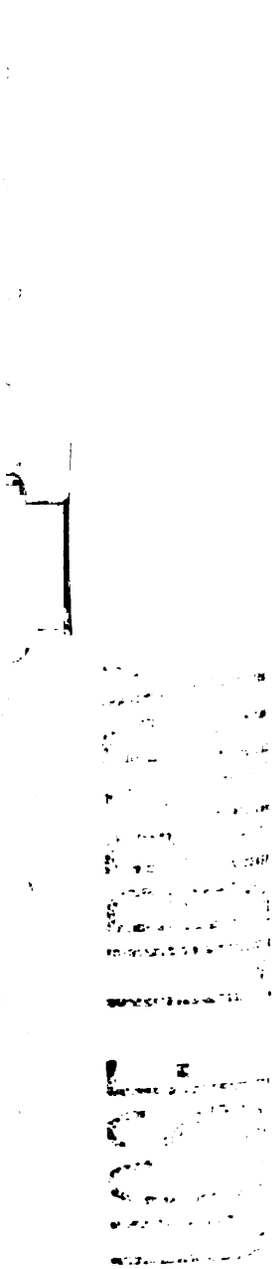


supported by genetic experiments showing a mislocalization of *osk* mRNA in *exu* null mutants. While further genetic analysis of Yps and the other proteins in the complex will provide additional insights into this problem, the current combination of biochemical cofractionation, *in vivo* colocalization, and genetic analysis argues for a direct role of Exu and its associated proteins in mRNA localizaton.

The role of the Exuperantia complex in mRNA localization

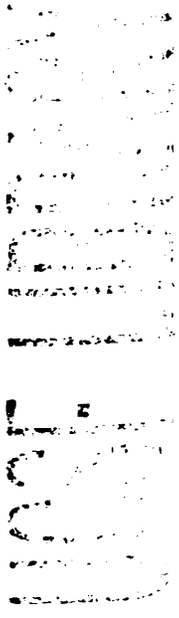
Previous genetic work implicated *exu* in mRNA localization, but did not address whether Exu plays a direct or indirect role in the localization process (Berleth et al., 1988; Macdonald et al., 1991; Marcey et al., 1991; St. Johnston et al., 1989). This study supports a direct involvement for Exu, since we show that Exu is part of a large RNAse sensitive complex and directly interacts with an RNA binding protein. Furthermore, the association of *osk* mRNA with the Exu complex establishes a physical connection between Exu and a localized mRNA. The biochemical characteristics of our large Exu complex also likely reflect the properties of Exu *in vivo*. Exu has been shown to be part of large particles by both GFP fluorescence (Wang and Hazelrigg, 1994) and immunoelectron microscopy (Wilsch-Brauninger et al., 1997). Moreover, Exu and Yps, which are associated components in our biochemically isolated complex, also colocalize in particles within the nurse cells and oocytes.

The identification of *osk* mRNA in the Exu-Yps complex was surprising, given the pronounced anterior patterning defects associated with



exu mutants (Schupbach and Wieschaus, 1986). However, a role for Exu in *osk* mRNA localization is consistent with several other findings. First, previous work has shown that Exu accumulates at both the anterior and posterior poles of the oocyte (Marcey et al., 1991; Wang and Hazelrigg, 1994). Second, *osk* mRNA transiently accumulates at the anterior pole along with *bcd* mRNA prior to its transport to the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991). Lastly, one of the effects of *exu* mutants was to disrupt the localization of *osk* mRNA to apical patches within nurse cells. This defect is identical to the nurse cell localization defect previously described for *bcd* mRNA in *exu* mutants (St. Johnston et al., 1989) and suggests that this step in the localization pathway may be common to both transcripts.

One of the reasons that *exu* mutants have not been previously examined for defects in *osk* mRNA localization is that only a small percentage of embryos from *exu* mothers display posterior patterning defects (Schupbach and Wieschaus, 1986). Our examination of *exu* mutants revealed that the amount of *osk* mRNA localized to the posterior pole is decreased in these mutants suggesting that *exu* plays role in localizing *osk* mRNA within oocytes. However, as this defect is only partially penetrant, Exu-dependent posterior localization within the oocyte may be redundant with other localization mechanisms. In addition, the posterior patterning defects associated with the decrease in *osk* mRNA localization in *exu* mutants during stages 9 and 10 of oogenesis may be rescued by localization of *osk* mRNA during cytoplasmic streaming later in oogenesis. In support of

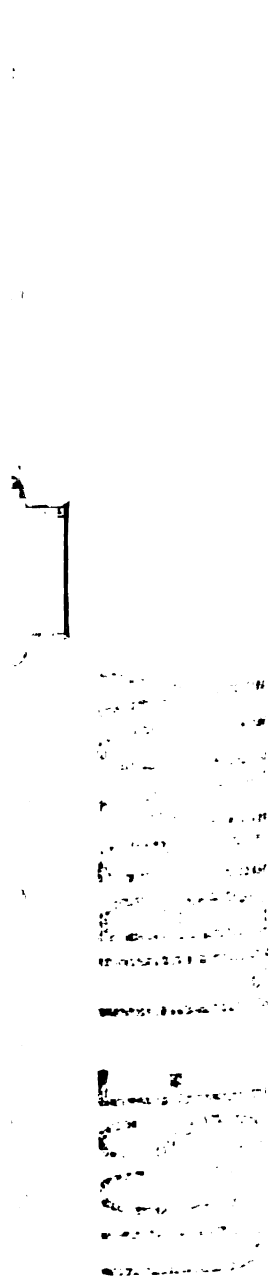


this idea, Glotzer *et. al.* (1997) have shown that injected, fluorescently labelled *osk* mRNA can be localized to the posterior at the time when cytoplasmic streaming occurs. Such localization most likely occurs by random motion during cytoplasmic streaming and specific anchoring of *osk* mRNAs that come in contact with the posterior pole. These multiple mechanisms of localizing *osk* mRNA account for the fact that *exu* mutants do not display pronounced defects in abdominal patterning.

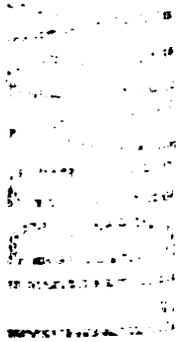
Previous work has shown that *exu* is necessary for normal *bcd* mRNA localization (Berleth et al., 1988; Macdonald and Kerr, 1997; Pokrywka and Stephenson, 1991; St. Johnston et al., 1989), although these studies did not establish a direct connection between Exu and *bcd* mRNA. Nevertheless, it was surprising that we could not detect *bcd* mRNA in our Exu complex. This negative result should not be taken to indicate that *bcd* mRNA is not in the complex, since it could reflect poor stability of *bcd* mRNA, a weaker association of *bcd* mRNA with the Exu complex, or difficulty in extracting/purifying RNP particles containing *bcd* mRNA. Alternatively, it is possible that *exu*'s role in *bcd* localization is indirect. We are currently trying to address these issues by employing other approaches to detect specific Exu-*bcd* mRNA interactions.

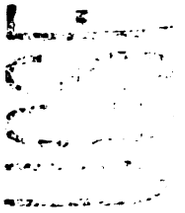
The role of Yps in mRNA localization

Our biochemical studies linking Yps to Exu and *osk* mRNA suggest that this RNA binding protein plays a role in posterior mRNA localization. This assertion is further supported by our immunofluorescence studies



showing that Yps and *osk* mRNA have strikingly similar localization patterns throughout oogenesis: both accumulate in the early oocyte, transiently localize to the oocyte anterior during stages 8/9 , and then assume their final positions at the posterior pole during stages 9/10 (Figure 8)(Ephrussi et al., 1991; Kim-Ha et al., 1991). What role might Yps play in the localization complex? Yps belongs to the cold shock domain family of RNA binding proteins that have been previously implicated in regulating translation and mRNA secondary structure (Jiang et al., 1997). A notable example is FRGY2, which is complexed with mRNAs in the *Xenopus* oocyte and is thought to be important for translational silencing (Tafuri and Wolffe, 1993; Yurkova and Murray, 1997). Yps may serve a similar role, since *osk* mRNA is translationally repressed until it reaches the posterior pole (Gunkel et al., 1998; Kim-Ha et al., 1995; Macdonald and Smibert, 1996). Interestingly, Yps must also serve a function without Exu, since *yps* is expressed broadly, while *exu* expression is limited to the germ line (Macdonald et al., 1991; Marcey et al., 1991; Thieringer et al., 1997). It is possible that Yps is a component of the mRNA localization machinery outside the germ line, since other components of the oocyte mRNA localization machinery, such as Staufen, are also utilized for mRNA localization in somatic tissues (Broadus et al., 1998; Li et al., 1997). Determining Yps' precise involvement in transport and/or translational regulation in the oocyte and other tissues will be resolved in the future by mutational studies.

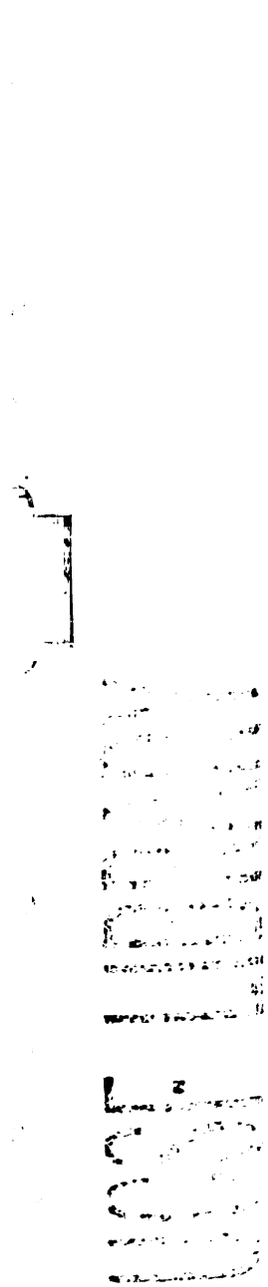




A model for mRNA transport by the Exu-Yps complex

The pathways by which anterior- and posterior-localized mRNAs arrive at their destinations are poorly understood, although it is generally believed that these RNAs are recognized by different proteins and utilize distinct transport machineries. We propose, however, that anterior- and posterior-localized mRNAs begin their localization process in the nurse cells using a similar complex, with Exu serving as a common core component (Fig. 10). In our model, one of Exu's functions is as a component of an mRNA transport complex, since GFP-Exu particles have been observed to move in a microtubule-dependent manner (Theurkauf and Hazelrigg, 1998). Consistent with this idea, both *osk* and *bcd* mRNA accumulate in apical patches within nurse cells, and *exu* mutants disrupt this localization pattern for both mRNAs (Ephrussi et al., 1991; Kim-Ha et al., 1991; St. Johnston et al., 1989)(Fig. 9B). We also propose that the Exu complex transports mRNAs from the nurse cells to the oocyte as well as within the oocyte (Fig. 10), although these transport steps also can be achieved through other redundant mechanisms, such as nurse cell dumping and cytoplasmic streaming (Spradling, 1993). While the above model places Exu as part of a transport complex, it should be noted that our present data also could be explained if Exu contributes to the establishment of anchoring once mRNAs reach their final destination.

After arriving in the oocyte, *bcd* and *osk*-containing RNPs must be sorted, so that *bcd* becomes anchored at the anterior while *osk* is transported



to the posterior pole. Since Yps, Exu, *bcd* mRNA and *osk* mRNA all first co-localize at the anterior (Figure 8)(Ephrussi et al., 1991; Kim-Ha et al., 1991; Theurkauf and Hazelrigg, 1998; Wang and Hazelrigg, 1994), we propose that this sorting decision occurs at the anterior of the oocyte (Figure 10). Evidence for this anterior sorting model comes from genetic studies of *staufen* (*stau*) and *tropomyosin II* (*TmII*) which show that these proteins do not interfere with anterior localization but rather block the release and transport of *osk* transcripts to the posterior (Ephrussi et al., 1991; Erdelyi et al., 1995; Kim-Ha et al., 1991; Tetzlaff et al., 1996). The molecular basis for this sorting decision is unclear, but may involve modifications to the transport machinery or the recruitment of additional factors.

Biochemistry of the Exu complex: relationship to *in vivo* function

Biochemical isolation of a native RNP complex provides an opportunity to identify new proteins involved in mRNA localization which may have been missed by genetic analyses due to lethality or redundant functions. A significant caveat of our approach, however, is that our isolated RNP particles may be heterogeneous, since our purification strategy begins with a crude extract containing material from egg chambers at all stages of oogenesis. Thus, it is likely that our biochemically-isolated material may represent a spectrum of "complexes" that comprise transport intermediates from the recognition of localized mRNAs in the nurse cells to the anchoring of the complex at the posterior pole. In addition to temporal changes in the

composition of the Exu complex, it is also possible that multiple Exu particles exist which contain distinct mRNA cargoes.

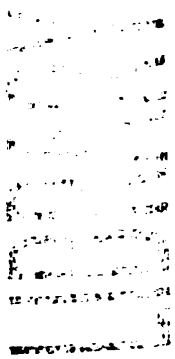
To resolve these important issues, it will be important to determine the molecular identity of the six RNAse-sensitive polypeptides in our Exu complex and determine if they are required selectively for the localization of *bcd* mRNA or *osk* mRNA. In addition, simultaneous observation and co-localization of GFP-tagged mRNAs (Bertrand et al., 1998) and BFP-tagged protein components of the Exu complex will reveal which mRNAs and polypeptides are contained within the same transport complex *in vivo*. This approach also will allow direct observation of mRNA transport in *Drosophila* for the first time and provide a method for analyzing the changes in RNP components during the movement of RNAs from the nurse cell to their final destination in the oocyte.

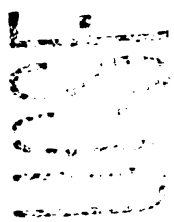
Acknowledgements

We thank Erin O'Shea, Joachim Li, and Vishu Lingappa for providing plasmids for *in vitro* translation studies, and Dave Morgan for providing the α-HA antibody. We also thank Paul MacDonald for generously providing the α-Exu antibody and much helpful advice. We are also grateful to Dara Friedman and Maki Inada for critically reading the manuscript and the members of the Vale lab for helpful discussions. This work was supported, in part, by grants from the National Institutes of Health to R.D.V. and T.H. J.E.W. was supported by the Medical Scientist Training Program.


A
9S
19S
30S
50S
B
RNase/Inhibitor
Inhibitor/RNAse
9S
19S
30S
50S

Figure 1: RNAse dependent sedimentation of Exu in *Drosophila* extracts. A) Sedimentation of Exu from an extract made from hand dissected ovaries through a 5-40% sucrose gradient (see Experimental Procedures). This immunoblot shows a broad distribution of Exu signal with the majority sedimenting greater than 20S. B) Sedimentation of Exu in extracts treated with RNAse A (10 μg/ml, 10 min at 4°C) followed by addition of pancreatic RNAse inhibitor (8000U/ml) (upper panel). As a control, RNAse A was premixed with the inhibitor prior to addition to the extract (lower panel). When RNAse A is added first, the Exu immunoblot signal is shifts from a large broad distribution to a tight 7S peak. While there is some variability in the sedimentation profile of Exu from extract to extract, there is always a significant fraction of Exu sedimenting at 20S or greater.





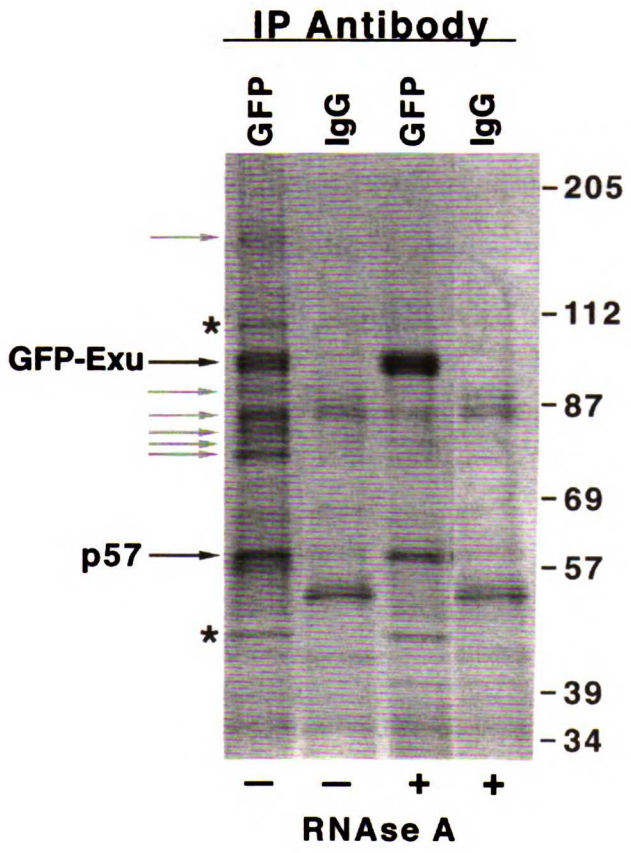

IP Antibody
GFP
IgG
GFP
IgG
205
112
87
69
57
39
34
GFP-Exu
p57
*
*
–
–
+
+
RNAse A

Figure 2: Immunoprecipitation of Exu-associated proteins.

Immunoprecipitations of GFP-Exu extracts were performed with either α–GFP antibodies or rabbit IgG. GFP-Exu and seven additional polypeptides (arrows) coimmunoprecipitate with α–GFP antibodies, but not with the rabbit IgG control. RNAse A treatment of the extracts during the immunoprecipitation shows that six of the associated polypeptides (grey arrows) require the presence of RNA to coimmunoprecipitate with GFP-Exu, while p57 (dark arrow) does not. Two additional proteins, p110 and p45, are also present in the immunoprecipitations (asterix). p110 only associates with Exu nonspecifically in the majority of immunoprecipitation reactions. p45 has been found to be a proteolytic fragment of p57 by microsequence analysis. Migration of molecular weight standards by SDS-PAGE are indicated on the right.

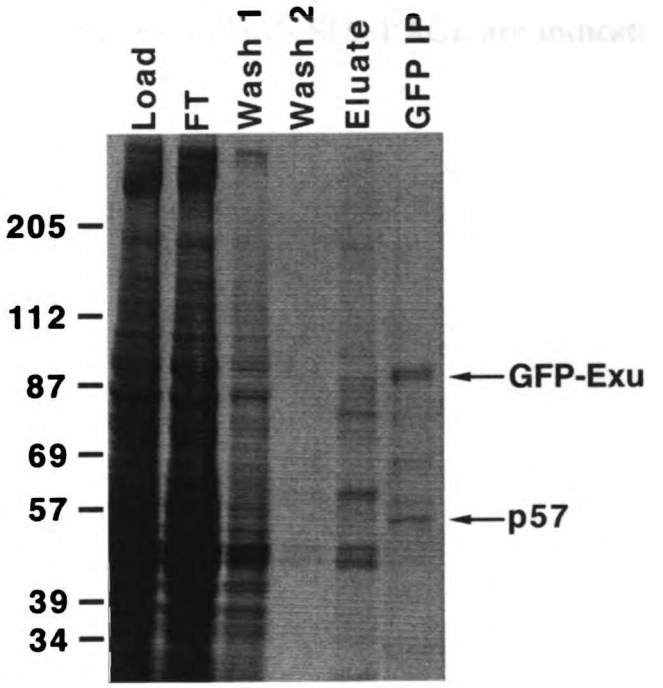

Load
FT
Wash 1
Wash 2
Eluate
GFP IP
205
112
87
69
57
39
34
GFP-Exu
p57

Figure 3: Purification of GFP-Exu-His$_6$ by metal affinity chromatography and GFP antibody immunoprecipitation. Shown are the initial load, the flow through (FT) from the Ni-NTA column, two wash steps, the imidazole eluate (Eluate), and α–GFP immunoprecipitation from the Ni-NTA eluate (GFP IP). p57 copurifies with GFP-Exu-His$_6$ through this procedure. Details of the purification procedures are found in the Experimental Procedures. Migration of molecular weight standards SDS-PAGE are indicated on the left.

A

```
MADAAESKPLAAEQQQQAQQQPEQQQNPPNPQEQDHEQEPLDELQGQQGQ  50
PAPPTKEVIATKVTGTVKWFNVKSGYGFINRNDTREDVFVHQSAIARNNP 100
KKAVRSVGDGEVVEFDVVIGEKGNEAANVTGPSgEPVRGSQFAADKRRNF 150
RPWMKKNRRKDGEVEGEDAESSAQQQQQQAAPIVDGQPQQQVQSGPRQPR 200
QNFRRGPPGGPPGGPRGGPRGPPGGAPGGPRRYNNYYLRQPRRGLGGGDG 250
SAEPGVHDQNPEGLQRGEGQGPRRGGGPPGGPQRRFFRRNFNNGPPPPRR 300
DGGEYIQGQGPPRPQQPRPRRQRKPNGPGGGSEQQPEKNgAqELQNTTTE 350
STA
```

B

```
 56 KEVIATKVTGTVKWFNVKSGYGFINRNDTREDVFVHQSAIARNNP  Drosophila Yps
 84 KKVLATKVTGTVKWFNVRnGYGFINRNDTKEDV              Human DBPA
 30 KKVLATKVlGTVKWFNVRnGYGFINRNDTKEDV              Xenopus YB1
 14 RKVLATGVeGTVKWFNVRnGYGFINRNDTKEDV              Goldfish YB2
 26 KKILASQVsGTVKWFNVKsGYGFINRDDTKEDV              Aplysia YB1

 89 FVHQSAIaRNNPRKAVRSVGDGEvVEFDVVIGE              Drosophila Yps
118 FVHQTAIkKNNPRKYLRSVGDGEtVEFDVVEGE              Human DBPA
 64 FVHQTAIkKNNPRKYLRSVGDGEtVEFDVVEGE              Xenopus YB1
 48 FVHQTAIkKNNPRKFLRSVGDGEvVEFDVVEAA              Goldfish YB2
 60 FVHQTAIvKNNPRKYLRSVGDGEkVEFDVVEGE              Aplysia YB1

123 KGnEAANVTGPsGePVrSGQFAADKRRNFR151              Drosophila Yps
152 KGaEAANVTGPdGvPVeGSRYAADRRRYRE179              Human DBPA
 98 KGaEAANVTGPeGvPVqGSKYAADRNHYRE125              Xenopus YB1
 82 KGsEAANVTGPgGiPVkGSRYAPNKRRFRE109              Goldfish YB2
 94 KGnEAANVTGPeGsNVqGSKYAADRRRFRE121              Aplysia YB1
```

Figure 4: Sequence of p57/Ypsilon Schactel, a cold shock domain RNA binding protein. A) The predicted protein sequence of Ypsilon Schactel determined from sequencing of 6 ESTs from the Berkeley *Drosophila* Genome Project. Solid lines highlight the peptides obtained by microsequencing of p57. Mismatches between the translated sequence and the microsequenced peptides are in lower case. The dashed line delineates the cold shock domain. Amino acid residue number are shown on the right. B) Sequence alignment of the cold shock domain of Yps with other cold shock domains from human (DBPA), Xenopus (YB1), goldfish (YB2), and Aplysia (YB1) proteins. Residues that are identical in all five proteins are highlighted. Residues that are identical or similar in four of the five proteins are shown in upper case and less well conserved residues are in lower case.

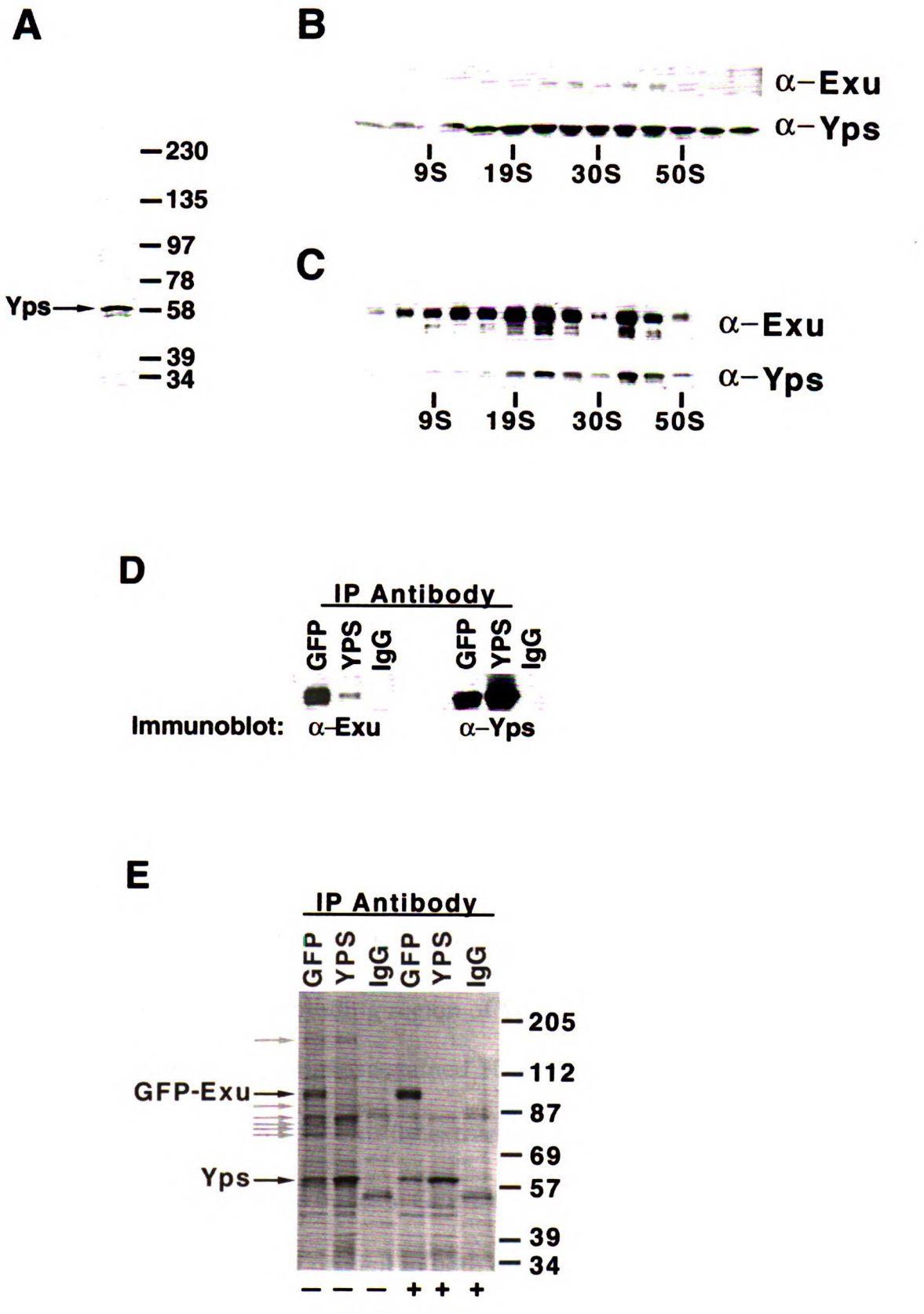

A
B
C
D
E
230
135
97
78
Yps
58
39
34
α–Exu
α–Yps
9S
19S
30S
50S
IP Antibody
GFP
YPS
IgG
Immunoblot:
α–Exu
α–Yps
GFP-Exu
Yps
205
112
87
69
57
39
34
RNAse A

Figure 5: Yps sedimentation and identification of associated proteins. A) Immunoblot of total fly extract with affinity purified α–Yps antibody. Migration of molecular weight standards by SDS-PAGE are indicated. B) Sedimentation of Exu and Yps in extracts made from frozen GFP-Exu expressing flies through a 5-40% sucrose gradient. Both Exu and Yps, detected by immunoblot, sediment broadly, with the peak centered at 25-30S. C) Immunoblot of Exu and Yps in α-GFP immunoprecipitations of a 5-40% sucrose density gradient loaded with extract made from frozen GFP-Exu expressing flies. Both Exu and Yps coimmunoprecipitate together across the gradient indicating that they are both part of the same complex. The decrease in signal in the fraction at 30S is due to poor recovery from that immunoprecipitation reaction. D) Immunoblot for Exu and Yps in immunoprecipitates from GFP-Exu extract using α-GFP (GFP), α-Yps (YPS), or rabbit IgG (IgG) antibodies. Exu coimmunoprecipitates with Yps, although the signal is not as robust as the Exu signal in the α-GFP immunoprecipitation. This is likely due to the fact that the α-Yps antibody was raised against the Exu binding region of Yps and hence, may displace Exu from the complex. E) Coomassise-stained SDS-PAGE gel of immunoprecipitates from GFP-Exu fly extracts using α-GFP, α-Yps, or rabbit IgG antibodies. Dark arrows indicate GFP-Exu and Yps. Grey arrows indicate the six polypeptides that are present in both α-GFP and α-Yps immunoprecipitates, but are absent from both immunoprecipitates when

RNAse-treated extracts are used (see Experimental Procedures). Migration of molecular weight standards by SDS-PAGE are indicated.

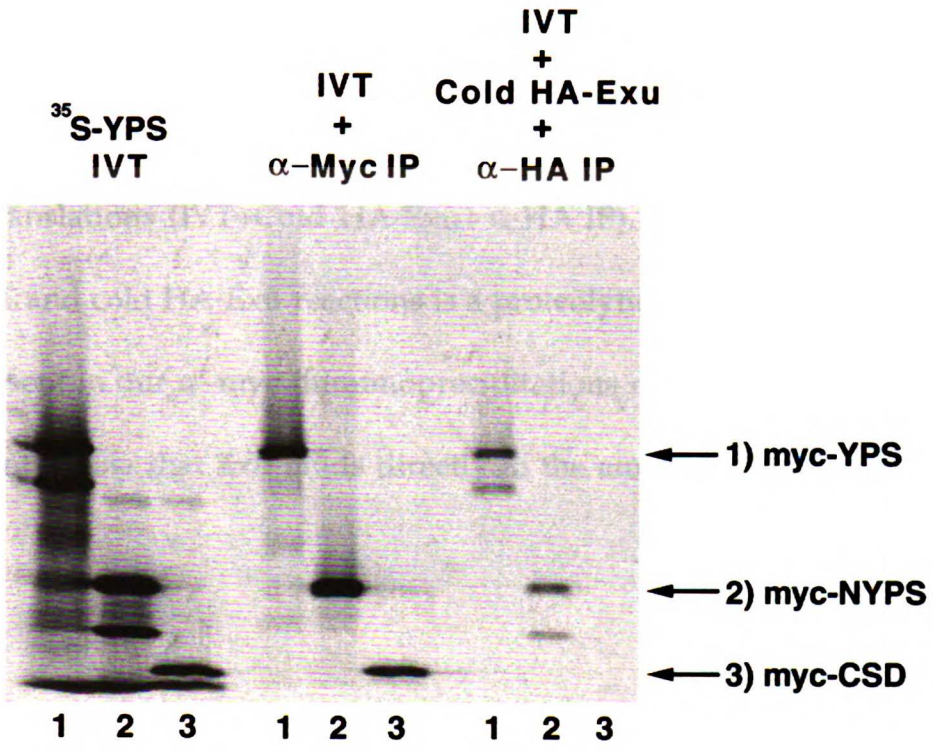

35S-YPS IVT
IVT + α–Myc IP
IVT + Cold HA-Exu + α–HA IP
1) myc-YPS
2) myc-NYPS
3) myc-CSD
1 2 3 1 2 3 1 2 3

Figure 6: Binding of Exu and Yps in *in vitro* translation reactions.

Autoradiogram of *in vitro* translation products of myc-tagged Yps (myc-YPS; lane 1), myc-tagged amino terminal Yps, amino acids 1-160, (myc-NYPS; lane 2), and myc-tagged cold shock domain of Yps, amino acids 56-151, (myc-CSD; lane 3). Shown are the *in vitro* translation products (^{35}S IVT), immunoprecipitations with the α-myc antibody (IVT+ α-myc IP), and α-HA immunoprecipitations of unlabelled HA-tagged Exu mixed with ^{35}S-labelled *in vitro* translations (IVT+Cold HA-Exu+ α-HA IP). The lower band present in the IVT and cold HA-Exu reactions is a proteolytic product of Yps, since it is not present in our α–myc immunoprecipitations of Yps. These experiments show that Exu binds directly to the amino terminal domain of Yps.

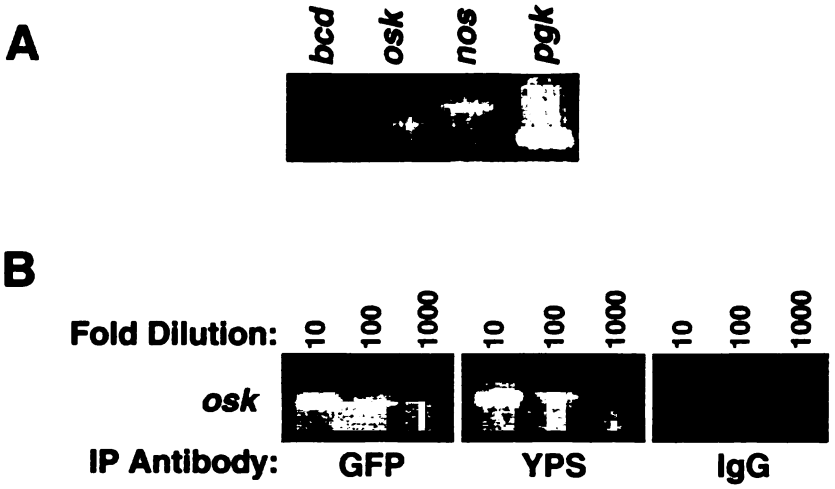

A
bcd
osk
nos
pgk
B
Fold Dilution:
10
100
1000
10
100
1000
10
100
1000
osk
IP Antibody:
GFP
YPS
IgG

Figure 7: Amplification of *oskar* mRNA by RT-PCR from the Exu-Yps complex. A) RT-PCR of *bicoid* (*bcd*), *oskar* (*osk*), *nanos* (*nos*), and *phosphoglycerokinase* (*pgk*) from 5 μg of total mRNA isolated from GFP-Exu extracts. All transcripts are easily detected in total mRNA. B) RT-PCR using *oskar* specific primers from RNA isolated from immunoprecipitates of GFP-Exu fly extract using α-GFP, α-Yps, or rabbit IgG. The reverse transcription reaction was diluted 10-, 100-, or 1000-fold, prior to the PCR step. In contrast to the signal observed with *osk* primers, *bcd*, *nos*, and *pgk* primers did not amplify any detectable signal from the same material (not shown). Details of this procedure are found in the Experimental Procedures.

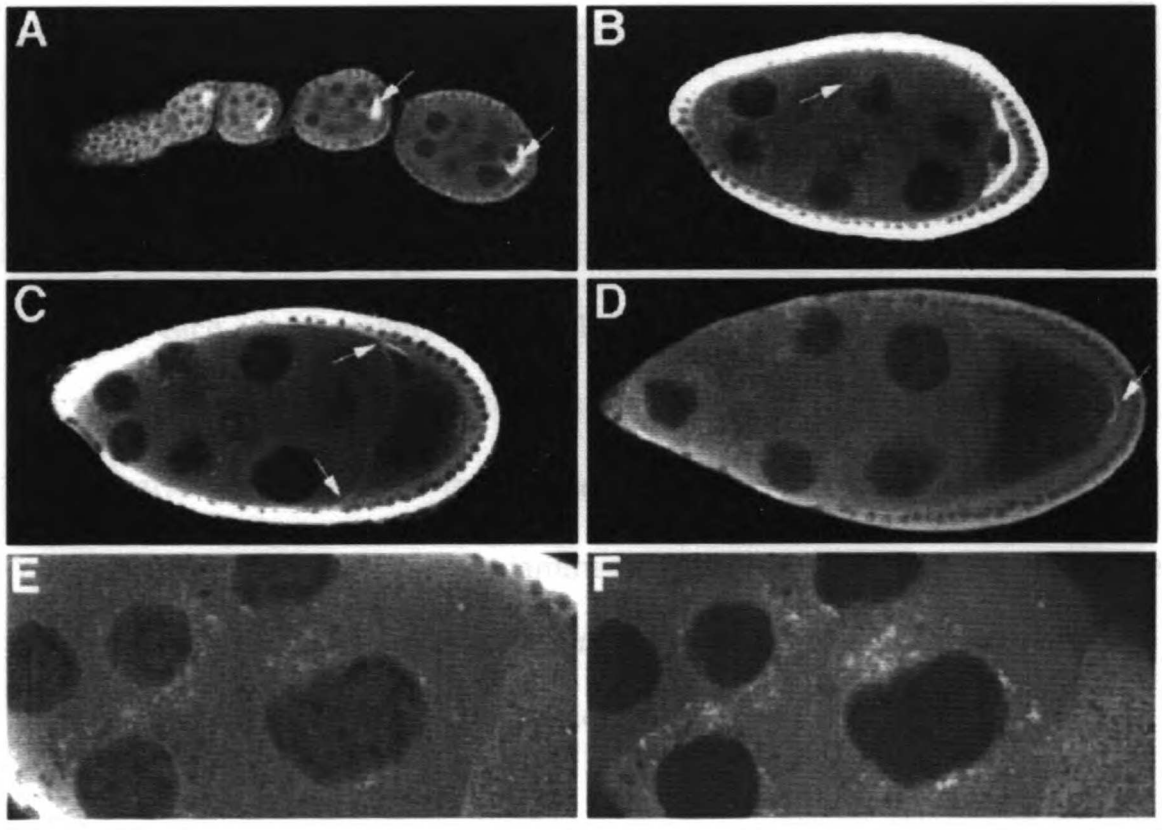

A
B
C
D
E
F

Figure 8: Yps accumulates at the posterior pole during mid-oogenesis and colocalizes with Exu within the ovary. Wild type (Oregon R) ovaries were labeled with α-Yps antibody (see Experimental Procedures). A) In previtellogenic stages, Yps is most concentrated in the oocyte (arrows). B) In both early and mid-stage egg chambers, Yps is present in particles that cluster around the nurse cell nuclei (arrow). C) At stage 9, Yps continues to be concentrated in particles in the nurse cell cytoplasm, while it begins to accumulate at the posterior pole and anterior margin (arrows) of the oocyte. D) By late stage 9, Yps is restricted to the posterior pole of the oocyte (arrow). Yps is also highly expressed in the follicle cells, the somatic cells that surround each egg chamber, during all stages of oogenesis, although this is not visible in all images due to the plane of focus. For colocalization studies (E,F), ovaries from flies carrying a *gfp-exu* transgene were labeled with an α-Yps antibody detected with a rhodamine-tagged secondary antibody. E) In the nurse cells of a stage 10 egg chamber, Yps is localized to perinuclear particles that colocalize with F) GFP-Exu particles.

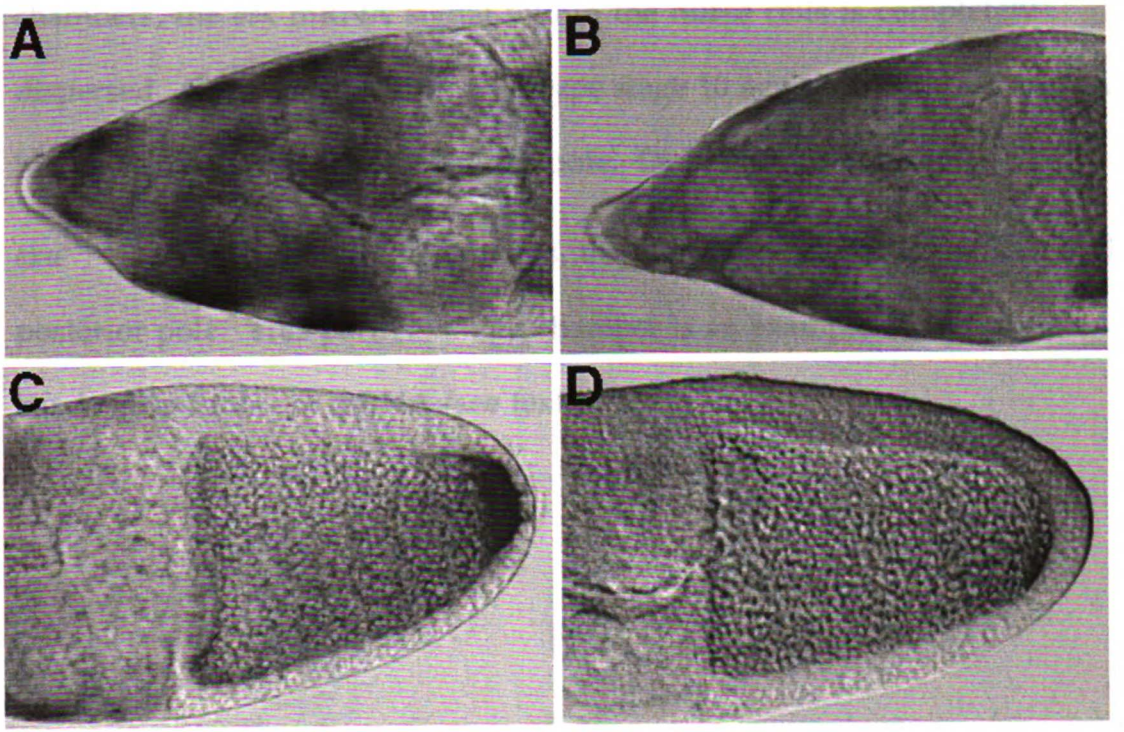

A
B
C
D

Figure 9: *oskar* mRNA localization is disrupted in *exu*^{SCO2} egg chambers. Whole mount *in situ* hybridization was performed on egg chambers from wild type (Oregon R) and *exu*^{SCO2} flies raised at 18°C to examine the distribution of *osk* mRNA. A) In the nurse cells of wild type stage 10 egg chambers, *osk* mRNA is often concentrated in patches within the nurse cell cytoplasm. B) In nurse cells from stage 10 *exu*^{SCO2} /Df(2R)MK1 egg chambers, the *osk* mRNA signal within the nurse cells is completely dispersed. C) In oocytes from wild type stage 10 egg chambers, *osk* mRNA is highly concentrated at the posterior pole. D) In oocytes from stage 10 *exu*^{SCO2} /Df(2R)MK1 egg chambers, the *osk* mRNA signal is reduced at the posterior pole. This particular egg chamber is an example of an oocyte with barely detectable *osk* mRNA (see text).

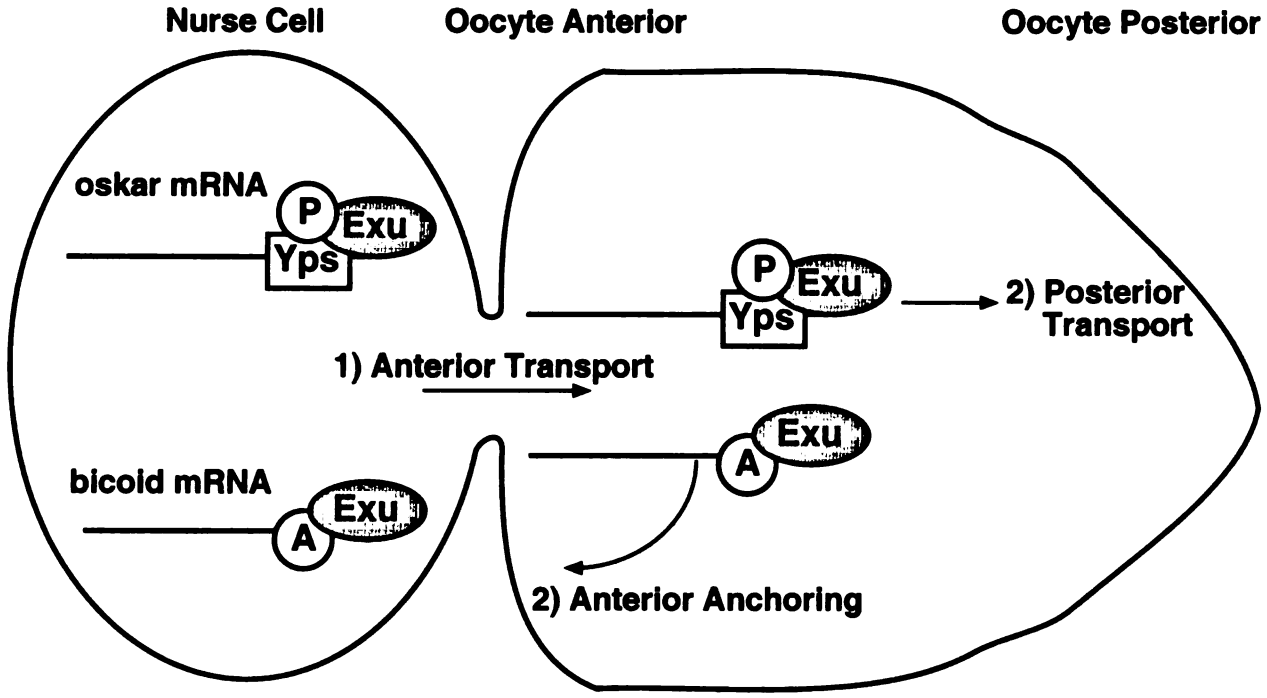

Nurse Cell
Oocyte Anterior
Oocyte Posterior
oskar mRNA
P
Exu
Yps
P
Exu
Yps
2) Posterior Transport
1) Anterior Transport
Exu
A
bicoid mRNA
Exu
A
2) Anterior Anchoring

Figure 10: A two step model for sorting mRNAs to the anterior and posterior poles of the oocyte. *oskar* and *bicoid* mRNAs each recruit transcript-specific proteins (A and P) as well as common components, such as Exu. Exu is involved in transport of *oskar* and *bicoid* mRNAs from the nurse cells to the anterior of the oocyte (Step 1). Once at the anterior, the specific components (A and P) sort mRNAs so that *bicoid* mRNA is anchored to the anterior of the oocyte, while *oskar* mRNA is transported to the posterior. In this model, we have depicted Yps as a candidate component of *oskar* mRNA specific complexes for reasons described in the text.

Future Directions

Our understanding of the mechanism of mRNA localization has been hindered by the inability to study the phenomenon in a system that is both genetically tractable and accessible to large scale biochemistry. One of the primary results of this thesis is that I have shown that mRNA transport in *Drosophila melanogaster* can now begin to be studied using a combination of genetic and biochemical approaches. Furthermore, the fact that two of the best characterized localized mRNAs, *oskar* and *bicoid*, appear to share some mechanistic components indicates that this system will be quite useful in addressing how mRNA localization is regulated to sort mRNAs to different locations during oogenesis.

The first step in achieving a mechanistic understanding of how mRNAs are targeted to particular subcellular locations is to identify all of the components of the transport complex for a particular localized mRNA. Isolation of the components of the Exu complex represents the first opportunity in any system to identify all of the protein components required for the localization of a particular message - the *oskar* mRNA. The fact that this biochemical analysis can be performed in an organism that is readily manipulated using molecular genetics should greatly facilitate the functional analysis of each component of the complex. The identification of Yps as a component of the Exu complex is the first step in this process. More recently, we have used mass spectrometry to identify two additional components of the Exu complex, Trailer Hitch and Winnebago (Appendix#1). While the analysis of these proteins is still ongoing, it is likely that they will yield

additional insights into the mechanism of mRNA localization. This prediction has already borne fruit in the case of the proteins, C64 and Oskar, which were also found to be present in the Exu complex by immunoblot analysis (Appendix#1). Since Oskar protein is only found at the posterior pole due to localization-dependent translational activation (Kim-Ha et al., 1995; Rongo et al., 1995) and C64 appears to be restricted to the nurse cells (T. Hazelrigg, personal communication), it is likely that the Exu "complex" I have isolated actually represents a population of Exu complexes that encompass all of the steps of the transport pathway from mRNA recognition to anchoring and translational activation. The biochemical analysis of mutants that block the various steps of *oskar* mRNA localization should help lay the groundwork for determining which components are part of the Exu complex during the various stages of the localization pathway and how the complex is altered to switch from one stage to the next.

The identification of the components of the mRNA localization machinery also will lay the groundwork for *in vitro* reconstitution of the various steps of the mRNA transport pathway. The establishment of such *in vitro* assays will allow us, for the first time, to assign specific biochemical functions to components of a mRNA transport complex. Since most models of the mechanism of mRNA transport have been limited by the fact that we have little biochemical understanding of the few proteins that have been implicated in transport, the development of these assays will allow us to fill in the gaps in existing models by determining how assembly of a large

ribonucleoprotein particle leads to transport along the cytoskeleton. One of the first steps in establishing this biochemical framework will be to determine how the Exu/Yps heterodimer specifically recognizes *oskar* mRNA. This question is particularly interesting since Exu is known to bind directly to C64, an RRM family RNA binding protein (T. Hazelrigg, personal communication). Does Exu bind to both Yps and C64 simultaneously or sequentially? How does the choice of binding partner affect the recruitment of Exu to a particular localized message? Answering these questions will help inform our views of how the transport complex is recruited and rearranged during localization.

Of course, the holy grail of these biochemical reconstitution experiments is to identify the component or components required to translocated messages along the cytoskeleton. Reconstitution of mRNA-based motility will allow us to determine which molecular motors are responsible for transporting mRNA and how these motors are targeted to their cargo. Even more importantly, however, such motility experiments will help to tease apart many of the redundant transport pathways that have confounded genetic analysis of mRNA localization in the past (Macdonald and Kerr, 1997) and will likely point the way to additional modes of cytoplasmic mRNA localization.

Because the process of mRNA localization is so poorly understood, most of the attention of researchers in the field has been focused on the mechanistic basis for mRNA transport, while relatively little attention has

been focused on regulatory issues, such as how transcripts are sorted to different regions of the cytoplasm and how the transport machinery is altered to perform specialized tasks in different tissues. These issues are likely to take center stage in the coming years as the components of the mRNA localization machinery become better defined. Even at this preliminary stage, our finding that Exu is required for both *bicoid* and *oskar* mRNA localization suggests that there may be a core transport complex that is altered or rearranged to sort transcripts to different locales within the cell. Furthermore, the recent finding that Exu exists in a large complex in males as well as females (Figure 1) and that *exu* mutants have both male and female sterile effects (Hazelrigg et al., 1990) suggests that mRNA localization may play a role in spermatogenesis in addition to its well characterized role in spermatogenesis. Since the proteins and RNAs that make up the male form of the Exu complex are unknown, determining the composition of this complex, the RNA cargoes it transports, and its role in spermatogenesis will help provide some of the first insights into how the mRNA localization machinery is adapted for tissue specific functions.

Clearly this is an exciting time for the field of mRNA localization as the components of the transport machinery are beginning to be characterized in a number of systems (Cote et al., 1999; Deshler et al., 1998; Havin et al., 1998; Hoek et al., 1998; Ross et al., 1997). However, the most interesting work lies ahead in determining how mRNA localization contributes processes as diverse as neuronal signalling, stem cell maintenance, and cell motility.

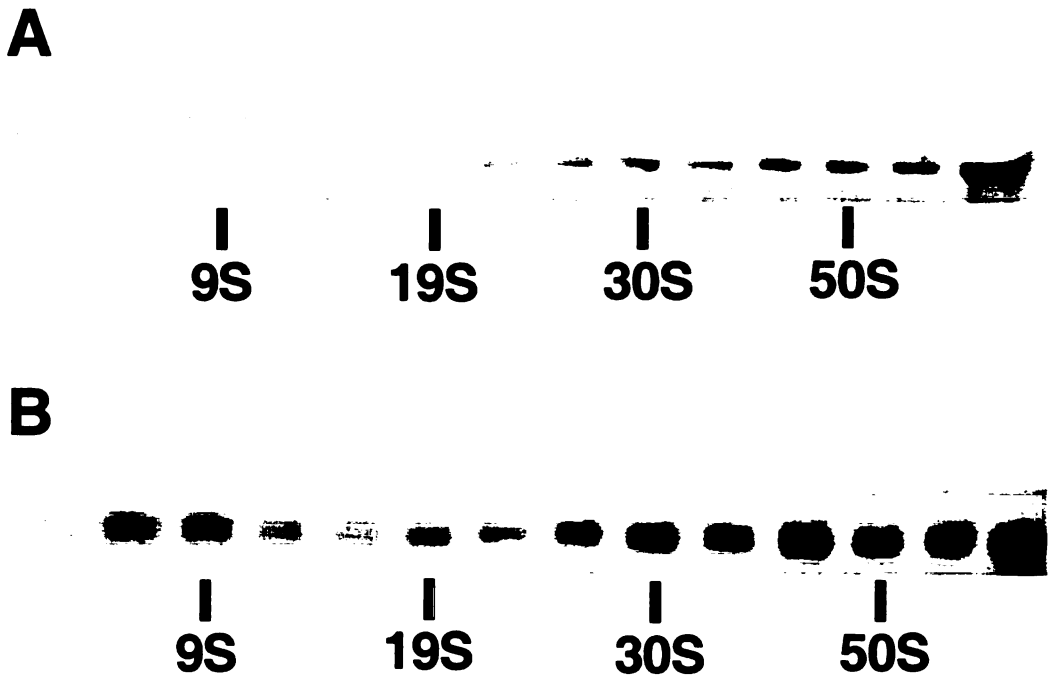

A
9S
19S
30S
50S
B
9S
19S
30S
50S

Figure 1: Exuperantia migrates as a large complex in extracts from both males and females. A) Sedimentation of Exu from an extract made from hand sorted male Drosophila through a 5-40% sucrose gradient (see Experimental Procedures). This immunoblot shows the majority of the Exu signal sedimenting greater than 20S. B) Sedimentation of Exu in extracts made from hand dissected ovaries through a 5-40% sucrose gradient. While the distribution of Exu signal is broader than in the male extract, there is always a significant fraction of Exu sedimenting at 20S or greater (see Chapter 3).

Appendix 1

As part of my ongoing characterization of the Exu complex, I have focused on identifying the 6 remaining polypeptides in the complex. To date, I have identified two additional components of the Exu by mass spectrometry in collaboration with Dr. Bill Henzel at Genentech. These two proteins, Trailer Hitch and Winnebago, specifically coimmunoprecipitate with both Exu and Yps (Figure 1) strongly indicating that both proteins are part of the Exu complex. Further studies demonstrating that Trailer Hitch and Winnebago co-peak with Exu and Yps on sucrose density gradients and that all 4 proteins co-localize *in vivo* are currently in progress.

Trailer Hitch is part of a large family of poorly characterized proteins that are present from yeast to plants to man. All family members have conserved amino and carboxy terminal domains that are separated by a low sequence complexity linker domain that varies in length from species to species (Figure 2). Of the family members that have been characterized, the most informative has been the *Pleurodeles waltl* (Newt) family member, RAP55. This protein is expressed only in the ovary and is part of a 40-120S RNAse-sensitive complex (Lieb et al., 1998). These findings are consistent with the observed behavior of the Exu complex on sucrose density gradients and suggest Trailer Hitch may be a broadly conserved component of the mRNA transport machinery. In order to confirm this, I have focused my efforts on characterizing the Saccharomyces cerevisiae homologue of Trailer Hitch, SCD6. SCD6 was originally identified as a high copy suppressor of clathrin heavy chain deficiency (S.K. Lemmon, unpublished observations).

SCD6 nulls have no observable growth defect and no previously described phenotype. My examination of ASH1 mRNA localization in SCD6 deficient strains revealed no defect in the transport of ASH1 mRNA to the daughter cell (data not shown). SCD6-GFP expressing strains only showed diffuse cytoplasmic fluorescence (data not shown); however, the functionality of the SCD6-GFP fusion protein cannot be addressed since there is no phenotype associated with the SCD6 deficiency. I am currently using microarray analysis to identify the RNAs associated with immunoprecipitates of both *Drosophila* Trailer Hitch and SCD6 in an effort to demonstrate an association between localized messages and members of the Trailer Hitch protein family. This approach should provide significant insights into the RNA cargoes for the Trailer Hitch family of proteins.

The Winnebago protein shares a number of sequence features with 5-formyltetrahydrofolate synthetases. These enzymes convert 5-formyltetrahydrofolate to 5,10-methenyltetrahydrofolate in an ATP dependent reaction. The 5-formyltetrahydrofolate synthetases contain no known ATP binding motifs and Winnebago does not contain any of the motifs implicated in substrate binding (Maras et al., 1994). Furthermore, a close relative of 5-formyltetrahydrofolate synthetase in other organisms was discovered by the *Drosophila* genome project indicating that Winnebago is not the *Drosophila* ortholog of this enzyme (unpublished observations). This sequence homology suggests that Winnebago may be a novel ATPase that plays a role in mRNA localization. I am currently attempting to test this

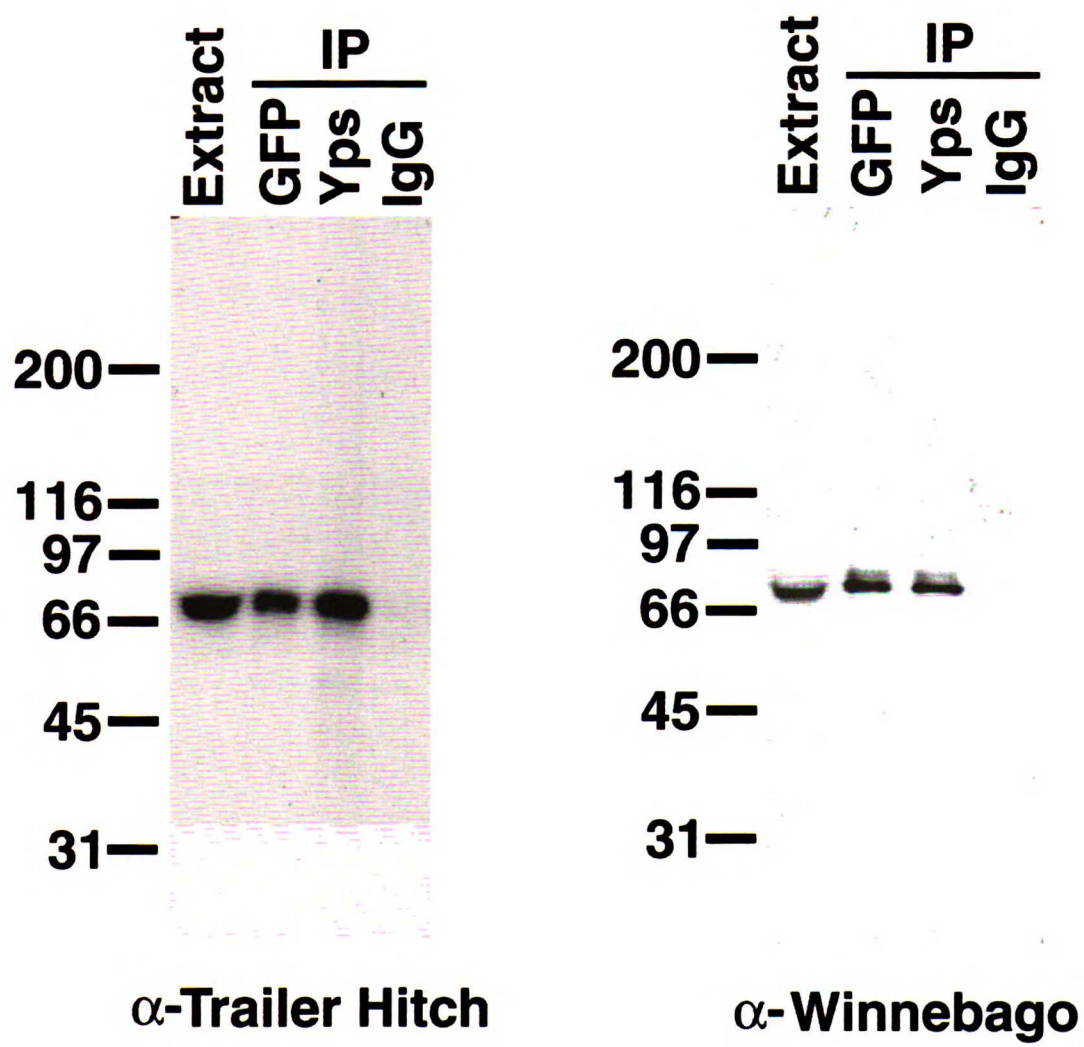
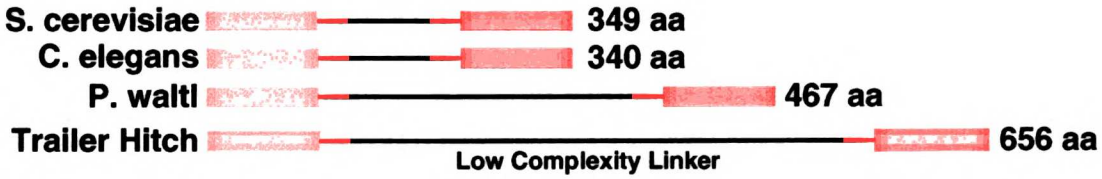

Extract
IP
GFP
Yps
IgG
200
116
97
66
45
31
α-Trailer Hitch
Extract
IP
GFP
Yps
IgG
200
116
97
66
45
31
α-Winnebago

Figure 1: Trailer Hitch and Winnebago specifically coimmunoprecipitate with both Exu and Yps. Immunoprecipitations of GFP-Exu extracts were performed with either α–GFP, α-Yps, or rabbit IgG antibodies. These immunoprecipitation reactions were immunoblotted for either Trailer Hitch (Left Panel) or Winnebago (Right Panel). In both cases, Trailer Hitch and Winnebago coimmunoprecipitated with Exu and Yps, but not with the IgG control.

A



B

```
Citrus          13 YIGsfISLISKyEIRYEGvLyhLn...vg DsTIgLknVKSFGTEgRkkdgpqv Lp.ser VYEYIlFRGSDIKDL
Medicago        15 YIGclISLtSKsEIRYEGvLynIn...td EsSIgLknVRSFGTEgRkkdgpqi Lp.gdk VYEYIlFRGTDIKDL
Neurospora       4 FLGsrISLISRsDIRYvGtLhnIn...se EsTvsLenVRSFGTEgRkhnpdeevpasdq VYEYIvFRGSDvKDL
S.pombe          4 FIGsrISLISKsDIRYvGiLqdIn...sq DsTLvLkhVRwcGTEgRkqdpsqe Ippsdn VFDYIvFRGSDvKDL
C.elegans        7 YIGskISLISKlDIRYEGiLytvd...tn DsTIaLakVRSFGTEkRptanp..vaardd VYEYIiFKaSDIKDL
Pleurodeles      7 YIGskISLISKaEIRYEGiLytId...tens TvvLakfall GTEdRptdrp.. Ipprde VFEYIiFRGSDIKDL
Drosophila       7 eLGskISLISKaDIRYEGrLytvd...pq EcTIaLssVRSFGTEdRdtqfq.. Iapqsqi YDYIlFRGSDIKDI
Dictyostelium    5 YIGakIvLISKslvKYEGiLytId...pk DnTLsLknVKSFGTEgRrqgtq.. Ippstei FDYIvFKsSDInDL
S.cerevisiae     4 YIGktISLISvtDnRYvGlLedId...sekg TvtLkeVRcFGTEgRknwgpee Iypnpt VYnsvkFnGSEvKDL
Schistosoma      8 iIGcpvSIISKakIRYEGnLhsIdfdtpn EpvItLskVwSFGTEdRpcerp..vaprnee YnqvvFRGrDLdDv
```

Figure 2: Trailer Hitch is a member of a broadly conserved family of proteins. A) The domain structures of Trailer Hitch family members from *S. cerevisiae, C. elegans, P. waltl,* and *D. melanogaster*. All the Trailer Hitch family members have conserved amino and carboxy terminal domains separated by a low sequence complexity linker domain of variable length. B) An alignment of the amino terminal domains of Trailer Hitch family members as well as sequences from ESTs for which the full length protein sequence is not known. The amino terminal domain of Trailer Hitch has homology to the translation products of ESTs from *Neurospora, Schistosoma, Citrus, Medicago,* and *Dictyostelium*. The identical amino acids are marked in yellow.

References

Ainger, K., Avossa, D., Morgan, F., Hill, S. J., Barry, C., Barbarese, E., and Carson, J. H. (1993). Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. J Cell Biol *123*, 431-441.

Andrews, D. W., Lauffer, L., Walter, P., and Lingappa, V. R. (1989). Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. J Cell Biol *108*, 797-810.

Bashirullah, A., Cooperstock, R. L., and Lipshitz, H. D. (1998). RNA localization in development. Annu Rev Biochem *67*, 335-394.

Bassell, G., and Singer, R. H. (1997). mRNA and cytoskeletal filaments. Curr Opin Cell Biol *9*, 109-115.

Bassell, G. J., Zhang, H., Byrd, A. L., Femino, A. M., Singer, R. H., Taneja, K. L., Lifshitz, L. M., Herman, I. M., and Kosik, K. S. (1998). Sorting of beta-actin mRNA and protein to neurites and growth cones in culture. J Neurosci *18*, 251-265.

Bergsten, S. E., and Gavis, E. R. (1999). Role for mRNA localization in translational activation but not spatial restriction of nanos RNA. Development *126*, 659-669.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M., and Nusslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. Embo J *7*, 1749-1756.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H., and Long, R. M. (1998). Localization of ASH1 mRNA particles in living yeast. Mol Cell *2*, 437-445.

Binder, R., Horowitz, J. A., Basilion, J. P., Koeller, D. M., Klausner, R. D., and Harford, J. B. (1994). Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. Embo J *13*, 1969-1980.

Bouvet, P., Matsumoto, K., and Wolffe, A. P. (1995). Sequence-specific RNA recognition by the Xenopus Y-box proteins. An essential role for the cold shock domain. J Biol Chem *270*, 28297-28303.

Breitwieser, W., Markussen, F. H., Horstmann, H., and Ephrussi, A. (1996). Oskar protein interaction with Vasa represents an essential step in polar granule assembly. Genes Dev *10*, 2179-2188.

Broadus, J., Fuerstenberg, S., and Doe, C. Q. (1998). Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. Nature *391*, 792-795.

Calnan, B. J., Biacalana, S., Hudson, D., and Frankel, A. D. (1991). Analysis of Arginine-Rich Peptides from the HIV TAT Protein Reveals Unusual Features of RNA-Protein Recognition. Genes Dev. *5*, 201-210.

Carey, J., Lowary, P. T., and Uhlenbeck, O. C. (1983). Interaction of R17 Coat Protein with Synthetic Variants of Its Ribonucleic Acid Binding Site. Biochemistry *22*, 4723-4730.

Carson, J. H., Worboys, K., Ainger, K., and Barbarese, E. (1997). Translocation of myelin basic protein mRNA in oligodendrocytes requires microtubules and kinesin. Cell Motil Cytoskeleton *38*, 318-328.

Chicurel, M. E., Singer, R. H., Meyer, C. J., and Ingber, D. E. (1998). Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. Nature *392*, 730-733.

Chung, C. T., Niemela, S. L., and Miller, R. H. (1989). One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci U S A *86*, 2172-2175.

Cote, C. A., Gautreau, D., Denegre, J. M., Kress, T. L., Terry, N. A., and Mowry, K. L. (1999). A Xenopus protein related to hnRNP I has a role in cytoplasmic RNA localization. Mol Cell *4*, 431-437.

Cripe, L., Morris, E., and Fulton, A. B. (1993). Vimentin mRNA location changes during muscle development. Proc Natl Acad Sci U S A *90*, 2724-2728.

Dahanukar, A., and Wharton, R. P. (1996). The Nanos gradient in Drosophila embryos is generated by translational regulation. Genes Dev *10*, 2610-2620.

Dale, L., Matthews, G., and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-beta-related domain of Xenopus Vg1. Embo J *12*, 4471-4480.

Dale, L., Matthews, G., Tabe, L., and Colman, A. (1989). Developmental expression of the protein product of Vg1, a localized maternal mRNA in the frog Xenopus laevis. Embo J *8*, 1057-1065.

Das, A. (1993). Control of Transcription Termination by RNA-Binding Proteins. Annu. Rev. Biochem. *62*, 893-930.

Deshler, J. O., Highett, M. I., Abramson, T., and 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., and Schnapp, B. J. (1997). Localization of Xenopus Vg1 mRNA by Vera protein and the endoplasmic reticulum. Science *276*, 1128-1131.

Driever, W., and Nusslein-Volhard, C. (1988). The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. Cell *54*, 95-104.

Driever, W., and Nusslein-Volhard, C. (1988). A gradient of bicoid protein in Drosophila embryos. Cell *54*, 83-93.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell *66*, 37-50.

Erdelyi, M., Michon, A. M., Guichet, A., Glotzer, J. B., and Ephrussi, A. (1995). Requirement for Drosophila cytoplasmic tropomyosin in oskar mRNA localization. Nature *377*, 524-527.

Ferrandon, D., Elphick, L., Nusslein-Volhard, C., and St Johnston, D. (1994). Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell *79*, 1221-1232.

Franklin, N. C. (1993). Clustered Arginine Residues of Bacteriophage λ N Protein are Essential to Antitermination of Transcription, but The Locale Cannot Compensate for boxB Loop Defects. J. Mol. Biol. *231*, 343-360.

Franklin, N. C. (1985). Conservation of Genome Form but Not Sequence in the Transcription Antitermination Determinants of Bacteriophages λ, ϕ21, and P22. J. Mol. Biol. *181*, 75-84.

Franklin, N. C. (1989). A Plasmid to Visualize and Assay Termination and Antitermination of Transcription in Escherichia coli. Plasmid *21*, 31-42.

Gavis, E. R. (1997). Expeditions to the pole: RNA localization in Xenopus and Drosophila. Trends in Cell Biology *7*, 485-492.

Gavis, E. R., Curtis, D., and Lehmann, R. (1996). Identification of cis-acting sequences that control nanos RNA localization. Dev Biol *176*, 36-50.

Gavis, E. R., and Lehmann, R. (1994). Translational regulation of nanos by RNA localization. Nature *369*, 315-318.

Gavis, E. R., Lunsford, L., Bergsten, S. E., and Lehmann, R. (1996). A conserved 90 nucleotide element mediates translational repression of nanos RNA. Development *122*, 2791-2800.

Glotzer, J. B., Saffrich, R., Glotzer, M., and Ephrussi, A. (1997). Cytoplasmic flows localize injected oskar RNA in Drosophila oocytes. Curr Biol *7*, 326-337.

Gonzalez, C. I., Ruiz-Echevarria, M. J., Vasudevan, S., Henry, M. F., and Peltz, S. W. (2000). Mol Cell 2:489-499.

Greenblatt, J., and Li, J. (1981). The NusA gene product of Escherichia coli: Its Identification and a demonstration that it interacts with the gene N transcription antitermination protein of bacteriophage lambda. J. Mol. Biol. *147*, 11-23.

Greenblatt, J., Nodwell, J. R., and Mason, S. W. (1993). Transcriptional Termination. Nature *364*, 401-406.

Gunkel, N., Yano, T., Markussen, F. H., Olsen, L. C., and Ephrussi, A. (1998). Localization-dependent translation requires a functional interaction between the 5' and 3' ends of oskar mRNA. Genes Dev *12*, 1652-1664.

Havin, L., Git, A., Elisha, Z., Oberman, F., Yaniv, K., Schwartz, S. P., Standart, N., and Yisraeli, J. K. (1998). RNA-binding protein conserved in both microtubule- and microfilament- based RNA localization. Genes Dev *12*, 1593-1598.

Hazelrigg, T. (1998). The destinies and destinations of RNAs. Cell *95*, 451-460.

Hazelrigg, T., Watkins, W. S., Marcey, D., Tu, C., Karow, M., and Lin, X. R. (1990). The exuperantia gene is required for Drosophila spermatogenesis as well as anteroposterior polarity of the developing oocyte, and encodes overlapping sex-specific transcripts. Genetics *126*, 607-617.

Heasman, J., Holwill, S., and Wylie, C. C. (1991). Fertilization of cultured Xenopus oocytes and use in studies of maternally inherited molecules. Methods Cell Biol *36*, 213-230.

Hegde, R. S., and Lingappa, V. R. (1996). Sequence-specific alteration of the ribosome-membrane junction exposes nascent secretory proteins to the cytosol. Cell *85*, 217-228.

Hellman, U., Wernstedt, C., Gonez, J., and Heldin, C. H. (1995). Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem *224*, 451-455.

Hoek, K. S., Kidd, G. J., Carson, J. H., and Smith, R. (1998). hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. Biochemistry *37*, 7021-7029.

Hyatt, B. A., Lohr, J. L., and Yost, H. J. (1996). Initiation of vertebrate left-right axis formation by maternal Vg1. Nature *384*, 62-65.

Hyatt, B. A., and Yost, H. J. (1998). The left-right coordinator: the role of Vg1 in organizing left-right axis formation. Cell *93*, 37-46.

Irish, V., Lehmann, R., and Akam, M. (1989). The Drosophila posterior-group gene nanos functions by repressing hunchback activity. Nature *338*, 646-648.

Isaacs, W. B., Cook, R. K., Van Atta, J. C., Redmond, C. M., and Fulton, A. B. (1989). Assembly of vimentin in cultured cells varies with cell type. J Biol Chem *264*, 17953-17960.

Isaacs, W. B., and Fulton, A. B. (1987). Cotranslational assembly of myosin heavy chain in developing cultured skeletal muscle. Proc Natl Acad Sci U S A *84*, 6174-6178.

Jeffery, W. R., Tomlinson, C. R., and Brodeur, R. D. (1983). Localization of actin messenger RNA during early ascidian development. Dev Biol *99*, 408-417.

Jiang, W., Hou, Y., and Inouye, M. (1997). CspA, the major cold-shock protein of Escherichia coli, is an RNA chaperone. J Biol Chem *272*, 196-202.

Kessler, D. S., and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. Development *121*, 2155-2164.

Kim-Ha, J., Kerr, K., and Macdonald, P. M. (1995). Translational regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. Cell *81*, 403-412.

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

Kim-Ha, J., Webster, P. J., Smith, J. L., and Macdonald, P. M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. Development *119*, 169-178.

Kislauskis, E. H., and Singer, R. H. (1992). Determinants of mRNA localization. Curr Opin Cell Biol *4*, 975-978.

Kjems, J., Calnan, B. J., Frankel, A. D., and Sharp, P. A. (1992). Specific Binding of a Basic Peptide from HIV-1 Rev. EMBO J. *11*, 1119-1129.

Klymkowsky, M. W., Maynell, L. A., and Nislow, C. (1991). Cytokeratin phosphorylation, cytokeratin filament severing and the solubilization of the maternal mRNA Vg1. J Cell Biol *114*, 787-797.

Kobayashi, S., Amikura, R., Nakamura, A., Saito, H., and Okada, M. (1995). Mislocalization of oskar product in the anterior pole results in ectopic localization of mitochondrial large ribosomal RNA in Drosophila embryos. Dev Biol *169*, 384-386.

Kobayashi, S., Goto, S., and Anzai, K. (1991). Brain-specific small RNA transcript of the identifier sequences is present as a 10 S ribonucleoprotein particle. J Biol Chem *266*, 4726-4730.

Lall, S., Francis-Lang, H., Flament, A., Norvell, A., Schupbach, T., and Ish-Horowicz, D. (1999). Squid hnRNP protein promotes apical cytoplasmic transport and localization of Drosophila pair-rule transcripts. Cell *98*, 171-180.

Lantz, V. A., Clemens, S. E., and Miller, K. G. (1999). The actin cytoskeleton is required for maintenance of posterior pole plasm components in the Drosophila embryo. Mech Dev *85*, 111-122.

Latham, V. M., Jr., Kislauskis, E. H., Singer, R. H., and Ross, A. F. (1994). Beta-actin mRNA localization is regulated by signal transduction mechanisms. J Cell Biol *126*, 1211-1219.

Lawrence, J. B., and Singer, R. H. (1986). Intracellular localization of messenger RNAs for cytoskeletal proteins. Cell *45*, 407-415.

Lazinski, D., Grzadzielska, E., and Das, A. (1989). Sequence-Specific Recognition of RNA Hairpins by Bacteriophage Antiterminators Requires a Conserved Arginine-Rich Motif. Cell *59*, 207-218.

Lehmann, R., and Nusslein-Volhard, C. (1991). The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development *112*, 679-691.

Li, P., Yang, X., Wasser, M., Cai, Y., and Chia, W. (1997). Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during Drosophila neuroblast cell divisions. Cell *90*, 437-447.

Lie, Y. S., and Macdonald, P. M. (1999). Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development *126*, 4989-4996.

Lieb, B., Carl, M., Hock, R., Gebauer, D., and Scheer, U. (1998). Identification of a novel mRNA-associated protein in oocytes of Pleurodeles waltl and Xenopus laevis. Exp Cell Res *245*, 272-281.

Lieberfarb, M. E., Chu, T., Wreden, C., Theurkauf, W., Gergen, J. P., and Strickland, S. (1996). Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. Development *122*, 579-588.

Long, R. M., Singer, R. H., Meng, X., Gonzalez, I., Nasmyth, K., and Jansen, R. P. (1997). Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. Science *277*, 383-387.

Lowary, P. T., and Uhlenbeck, O. C. (1987). An RNA Mutation That Increases the Affinity of an RNA-Protein Interaction. Nucleic Acids Res. *15*, 10483-10493.

MacDonald, P. M. (1990). bicoid mRNA localization signal: phylogenetic conservation of function and RNA secondary structure. Development *110*, 161-171.

Macdonald, P. M., and Kerr, K. (1997). Redundant RNA recognition events in bicoid mRNA localization. Rna *3*, 1413-1420.

MacDonald, P. M., Leask, A., and Kerr, K. (1995). exl protein specifically binds BLE1, a bicoid mRNA localization element, and is required for one phase of its activity. Proc Natl Acad Sci U S A *92*, 10787-10791.

Macdonald, P. M., Luk, S. K., and Kilpatrick, M. (1991). Protein encoded by the exuperantia gene is concentrated at sites of bicoid mRNA accumulation in Drosophila nurse cells but not in oocytes or embryos. Genes Dev *5*, 2455-2466.

Macdonald, P. M., and Smibert, C. A. (1996). Translational regulation of maternal mRNAs. Curr Opin Genet Dev *6*, 403-407.

MacWilliams, M. P., Celander, D. W., and Gardner, J. F. (1993). Direct genetic selection for a specific RNA-protein interaction. Nucleic Acids Res *21*, 5754-5760.

Madhani, H. D., and Guthrie, C. (1994). Randomization-selection analysis of snRNAs in vivo: evidence for a tertiary interaction in the spliceosome. Genes Dev *8*, 1071-1086.

Maras, B., Stover, P., Valiante, S., Barra, D., and Schirch, V. (1994). Primary structure and tetrahydropteroylglutamate binding site of rabbit liver cytosolic 5,10-methenyltetrahydrofolate synthetase. J Biol Chem *269*, 18429-18433.

Marcey, D., Watkins, W. S., and Hazelrigg, T. (1991). The temporal and spatial distribution pattern of maternal exuperantia protein: evidence for a role in establishment but not maintenance of bicoid mRNA localization. Embo J *10*, 4259-4266.

Mason, S. W., Li, J., and Greenblatt, J. (1992). Host Factor Requirements for Processive Antitermination of Transcription and Suppression of Pausing by the N protein of Bacteriophage λ. J. Biol. Chem. *267*, 19418-19426.

Matsumoto, K., Meric, F., and Wolffe, A. P. (1996). Translational repression dependent on the interaction of the Xenopus Y- box protein FRGY2 with mRNA. Role of the cold shock domain, tail domain, and selective RNA sequence recognition. J Biol Chem *271*, 22706-22712.

McDonald, D., Hope, T. J., and Parslow, T. G. (1992). Posttranscriptional regulation by the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex proteins through a heterologous RNA binding site. J Virol *66*, 7232-7238.

Melton, D. A. (1987). Translocation of a localized maternal mRNA to the vegetal pole of Xenopus oocytes. Nature *328*, 80-82.

Micklem, D. R., Dasgupta, R., Elliott, H., Gergely, F., Davidson, C., Brand, A., Gonzalez-Reyes, A., and St. Johnston, D. (1997). The mago nashi gene is required for the polarisation of the oocyte and the formation of perpendicular axes in Drosophila. Curr Biol *7*, 468-478.

Miller, J. H. (1992). . In A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria (Plainview, NY: Cold Spring Harbor Laboratory Press), pp. 72-74.

Moroianu, J. (1999). Nuclear import and export: transport factors, mechanisms and regulation. Crit Rev Eukaryot Gene Expr *9*, 89-106.

Mosquera, L., Forristall, C., Zhou, Y., and King, M. L. (1993). A mRNA localized to the vegetal cortex of Xenopus oocytes encodes a protein with a nanos-like zinc finger domain. Development *117*, 377-386.

Mowry, K. L., and Melton, D. A. (1992). Vegetal messenger RNA localization directed by a 340-nt RNA sequence element in Xenopus oocytes. Science *255*, 991-994.

Murray, M. T. (1994). Nucleic acid-binding properties of the Xenopus oocyte Y box protein mRNP3+4. Biochemistry *33*, 13910-13917.

Neupert, W. (1997). Protein import into mitochondria. Annu Rev Biochem *66*, 863-917.

Nodwell, J. R., and Greenblatt, J. (1991). The nut site of Bacteriophage l is Made of RNA and is bound by Transcription Antitermination Factors on the surface of RNA Polymerase. Genes Dev. *5*, 2141-2151.

Nodwell, J. R., and Greenblatt, J. (1993). Recognition of boxA Antiterminator RNA by the E. coli Antitermination Factors NusB and the Ribosomal Protein S10. Cell *72*, 261-268.

Oleynikov, Y., and Singer, R. H. (1998). RNA localization: different zipcodes, same postman? Trends Cell Biol *8*, 381-383.

Ostareck, L. A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994). Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. Embo J *13*, 1476-1481.

Pokrywka, N. J., and Stephenson, E. C. (1994). Localized RNAs are enriched in cytoskeletal extracts of Drosophila oocytes. Dev Biol *166*, 210-219.

Pokrywka, N. J., and Stephenson, E. C. (1995). Microtubules are a general component of mRNA localization systems in Drosophila oocytes. Dev Biol *167*, 363-370.

Pokrywka, N. J., and Stephenson, E. C. (1991). Microtubules mediate the localization of bicoid RNA during Drosophila oogenesis. Development *113*, 55-66.

Pondel, M. D., and King, M. L. (1988). Localized maternal mRNA related to transforming growth factor beta mRNA is concentrated in a cytokeratin-enriched fraction from Xenopus oocytes. Proc Natl Acad Sci U S A *85*, 7612-7616.

Rapoport, T. A. (1990). Protein transport across the ER membrane. Trends Biochem Sci *15*, 355-358.

Rebagliati, M. R., Weeks, D. L., Harvey, R. P., and Melton, D. A. (1985). Identification and cloning of localized maternal RNAs from Xenopus eggs. Cell *42*, 769-777.

Romaniuk, P. J., Lowary, P., Wu, H. N., Stormo, G., and Uhlenbeck, O. C. (1987). RNA Binding Site of R17 Coat Protein. Biochemistry *26*, 1563-1568.

Rongo, C., Gavis, E. R., and Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development *121*, 2737-2746.

Ross, A. F., Oleynikov, Y., Kislauskis, E. H., Taneja, K. L., and Singer, R. H. (1997). Characterization of a beta-actin mRNA zipcode-binding protein. Mol Cell Biol *17*, 2158-2165.

Salles, F. J., Lieberfarb, M. E., Wreden, C., Gergen, J. P., and Strickland, S. (1994). Coordinate initiation of Drosophila development by regulated polyadenylation of maternal messenger RNAs. Science *266*, 1996-1999.

Saunders, C., and Cohen, R. S. (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.

Schneider, D., Tuerk, C., and Gold, L. (1992). Selection of High Affinity RNA Ligansd to the Bacteriophage R17 Coat Protein. J. Mol. Biol. *228*, 862-869.

Schupbach, T., and Wieschaus, E. (1986). Maternal effect mutations altering the anterior-posterior pattern of the Drosophila embryo. Wilhelm Roux Archives of Developmental Biology *195*, 302-317.

Schwartz, S. P., Aisenthal, L., Elisha, Z., Oberman, F., and Yisraeli, J. K. (1992). A 69-kDa RNA-binding protein from Xenopus oocytes recognizes a common motif in two vegetally localized maternal mRNAs. Proc Natl Acad Sci U S A *89*, 11895-11899.

Selby, M. J., and Peterlin, B. M. (1990). Trans-activation by HIV-1 Tat via a heterologous RNA binding protein. Cell *62*, 769-776.

Sheets, M. D., Fox, C. A., Hunt, T., Vande Woude, G., and Wickens, M. (1994). The 3'-untranslated regions of c-mos and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev *8*, 926-938.

Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of oskar directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. Cell *70*, 849-859.

Smith, L. D., Xu, W. L., and Varnold, R. L. (1991). Oogenesis and oocyte isolation. Methods Cell Biol *36*, 45-60.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In The Development of Drosophila melanogaster (Plainview, N.Y.: Cold Spring Harbor Laboratory Press), pp. 1-70.

Spradling, A. C., and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. Science *218*, 341-347.

St Johnston, D. (1995). The intracellular localization of messenger RNAs. Cell *81*, 161-170.

St Johnston, D., Beuchle, D., and Nusslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the Drosophila egg. Cell *66*, 51-63.

St Johnston, D., and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. Cell *68*, 201-219.

St. Johnston, D., Driever, W., Berleth, T., Richstein, S., and Nusslein-Volhard, C. (1989). Multiple steps in the localization of bicoid RNA to the anterior pole of the Drosophila oocyte. Development *107*, 13-19.

Stephenson, E. C., Chao, Y. C., and Fackenthal, J. D. (1988). Molecular analysis of the swallow gene of Drosophila melanogaster. Genes Dev *2*, 1655-1665.

Stripecke, R., Oliveira, C. C., McCarthy, J. E., and Hentze, M. W. (1994). Proteins binding to 5' untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. Mol Cell Biol *14*, 5898-5909.

Sundell, C. L., and Singer, R. H. (1991). Requirement of microfilaments in sorting of actin messenger RNA. Science *253*, 1275-1277.

Tafuri, S. R., and Wolffe, A. P. (1993). Selective recruitment of masked maternal mRNA from messenger ribonucleoprotein particles containing FRGY2 (mRNP4). J Biol Chem *268*, 24255-24261.

Takizawa, P. A., Sil, A., Swedlow, J. R., Herskowitz, I., and Vale, R. D. (1997). Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. Nature *389*, 90-93.

Taneja, K. L., Lifshitz, L. M., Fay, F. S., and Singer, R. H. (1992). Poly(A) RNA codistribution with microfilaments: evaluation by in situ hybridization and quantitative digital imaging microscopy. J Cell Biol *119*, 1245-1260.

Tannahill, D., and Melton, D. A. (1989). Localized synthesis of the Vg1 protein during early Xenopus development. Development *106*, 775-785.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma *98*, 81-85.

Tetzlaff, M. T., Jackle, H., and Pankratz, M. J. (1996). Lack of Drosophila cytoskeletal tropomyosin affects head morphogenesis and the accumulation of oskar mRNA required for germ cell formation. Embo J *15*, 1247-1254.

Theurkauf, W. E., and Hazelrigg, T. I. (1998). In vivo analyses of cytoplasmic transport and cytoskeletal organization during Drosophila oogenesis: characterization of a multi-step anterior localization pathway. Development *125*, 3655-3666.

Theurkauf, W. E., Smiley, S., Wong, M. L., and Alberts, B. M. (1992). Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development *115*, 923-936.

Thieringer, H. A., Singh, K., Trivedi, H., and Inouye, M. (1997). Identification and developmental characterization of a novel Y-box protein from Drosophila melanogaster. Nucleic Acids Res *25*, 4764-4770.

Thomsen, G. H., and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. Cell *74*, 433-441.

Tiedge, H., Fremeau, R. T., Jr., Weinstock, P. H., Arancio, O., and Brosius, J. (1991). Dendritic location of neural BC1 RNA. Proc Natl Acad Sci U S A *88*, 2093-2097.

Tiley, L. S., Madore, S. J., Malim, M. H., and Cullen, B. R. (1992). The VP16 transcription activation domain is functional when targeted to a promoter-proximal RNA sequence. Genes Dev *6*, 2077-2087.

Valegard, K., Murray, J. B., Stockley, P. G., Stonehouse, N. J., and Liljas, L. (1994). Crystal structure of an RNA bacteriophage coat protein-operator complex. Nature *371*, 623-626.

Venkatesan, S., Gerstberger, S. M., Park, H., Holland, S. M., and Nam, Y. (1992). Human immunodeficiency virus type 1 Rev activation can be achieved without Rev-responsive element RNA if Rev is directed to the

target as a Rev/MS2 fusion protein which tethers the MS2 operator RNA. J Virol *66*, 7469-7480.

Wang, S., and Hazelrigg, T. (1994). Implications for bcd mRNA localization from spatial distribution of exu protein in Drosophila oogenesis. Nature *369*, 400-403.

Webster, P. J., Liang, L., Berg, C. A., Lasko, P., and Macdonald, P. M. (1997). Translational repressor bruno plays multiple roles in development and is widely conserved. Genes Dev *11*, 2510-2521.

Weeks, K. M., Ampe, C., Schultz, S. C., Steitz, T. A., and Crothers, D. M. (1990). Fragments of HIV-1 TAT Protein Specifically Bind TAR RNA. Science *249*, 1281-1285.

Whalen, W., Ghosh, B., and Das, A. (1988). NusA protein is necessary and sufficient in vitro for phage λ N gene product to suppress a ρ-independent terminator placed downstream of nutL. Proc. Natl. Acad. Sci. USA *85*, 2494-2498.

Wharton, R. P., and Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen nanos. Cell *67*, 955-967.

Wilhelm, J. E., and Vale, R. D. (1993). RNA on the move: the mRNA localization pathway. J Cell Biol *123*, 269-274.

Wilsch-Brauninger, M., Schwarz, H., and Nusslein-Volhard, C. (1997). A sponge-like structure involved in the association and transport of maternal products during Drosophila oogenesis. J Cell Biol *139*, 817-829.

Yisraeli, J. K., and Melton, D. A. (1988). The material mRNA Vg1 is correctly localized following injection into Xenopus oocytes. Nature *336*, 592-595.

Yisraeli, J. K., Sokol, S., and Melton, D. A. (1989). The process of localizing a maternal messenger RNA in Xenopus oocytes. Development *107*, 31-36.

Yisraeli, J. K., Sokol, S., and Melton, D. A. (1990). A two-step model for the localization of maternal mRNA in Xenopus oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA. Development *108*, 289-298.

Yurkova, M. S., and Murray, M. T. (1997). A translation regulatory particle containing the Xenopus oocyte Y box protein mRNP3+4. J Biol Chem *272*, 10870-10876.

Zhang, H. L., Singer, R. H., and Bassell, G. J. (1999). Neurotrophin regulation of beta-actin mRNA and protein localization within growth cones. J Cell Biol *147*, 59-70.