Inhibition of Xnos1 Translation by Structural Elements in the Open Reading Frame

Stephen Tyler Nerlick
University of Miami, snerlick@hotmail.com

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INHIBITION OF XNOS1 TRANSLATION BY STRUCTURAL ELEMENTS IN THE OPEN READING FRAME

By

Stephen Nerlick

A THESIS

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INHIBITION OF XNOS1 TRANSLATION BY STRUCTURAL ELEMENTS IN THE OPEN READING FRAME

Stephen Nerlick

Approved:

Dr. Arun Malhotra
Associate Professor of Biochemistry

Dr. Terri A. Scandura
Dean of the Graduate School

Dr. Mary Lou King
Professor of Cell Biology

Dr. Roland Jurecic
Associate Professor of Microbiology

Dr. Murray Deutscher
Professor of Biochemistry
NERLICK, STEPHEN (M.S., Biochemistry)

Inhibition of Xnos1 Translation by Structural Elements in the Open Reading Frame (June 2008)

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The spatio-temporal regulation of translation is critical to the proper development of all organisms. The presence of Translational Control Elements (TCEs) in Un-Translated Regions (UTRs) is one feature common to all regulated eukaryotic mRNAs examined to date. These TCEs serve as binding sites for sequence specific proteins or small regulatory RNAs that recruit other accessory proteins that inhibit translation. Xnos1, a localized RNA in the germ plasm of *Xenopus*, is negatively regulated by an unknown mechanism. We used *in vivo* and *in vitro* translation assays, competition assays, and ribosome binding assays in order to determine the location of the Xnos1 TCE and its mechanism of repression. The Xnos1 TCE is located in the open reading frame, a departure from the canonical translational regulation mechanisms. This TCE is predicted to form conserved secondary structures in the first 75 nucleotides of the open reading frame (ORF). *In vitro* translation assays demonstrated that the repression can be partially relieved by either denaturing the transcripts or by introducing point mutations that weaken the secondary structure. This TCE cannot be relieved by competition and therefore is likely not to require the presence of a repressor protein. The structural regulation of translation by a TCE in the open reading frame is a novel mechanism of repression for a eukaryotic mRNA.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>List/Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2 RESULTS</td>
<td>5</td>
</tr>
<tr>
<td>3 FIGURES</td>
<td>14</td>
</tr>
<tr>
<td>4 DISCUSSION</td>
<td>27</td>
</tr>
<tr>
<td>5 MATERIALS AND METHODS</td>
<td>32</td>
</tr>
<tr>
<td>References</td>
<td>39</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. TCE of Xnos1 is located in the ORF. 14
Figure 2. A conserved secondary structure predicted in the first 75nt of the Xnos1 ORF. 15
Figure 3. Denaturing relieves Xnos1 repression in oocyte extracts. 16
Figure 4. Context dependence of Myc-Xnos1 fusion constructs. 17
Figure 5. Competition does not relieve Xnos1 repression. 18
Figure 6. Early stage embryos translate Xnos1-Myc transcripts. 19
Figure 7. Xnos1-Myc does not bind the 40S subunit. 20
Figure 8. GMP-PNP causes increased binding of Myc-Xnos1 to 40S subunit. 21
Figure 9. EDTA is required to separate ribosome into subunits. 22
Figure 10. EDTA in excess of Mg2+ is required to separate ribosome into subunits. 23
Figure 11. Ribosome subunit isolation. 24
Figure 12. Model for Xnos1 translational repression. 26
Chapter 1: Introduction

Embryonic development is dependant on the tightly regulated translation of transcripts stored in the egg prior to fertilization. The regulated translation of these stored maternal transcripts is critical because these genes control cell cycle and cell fate decisions (Zhang et al., 1998; Zhang and Bienz, 1992). The mechanisms of translational repression are as varied as the genes, but several common themes have emerged. These themes center on binding of repressor proteins or short RNAs to specific sequences, called Translational Control Elements (TCEs), found in the 5’ or 3’ UTRs. These binding events repress translation by inhibiting initiation events at the 5’ cap, regulating poly-A tail length, or destabilizing the transcript (Johnstone and Lasko, 2001; Schier, 2007).

Inhibitors of initiation events target the eIF4E-eIF4G or the eIF4E-cap interaction (Richter and Sonenberg, 2005). Eukaryotic translation starts with the circularization of the transcript by association of eIF4E, which binds the cap, and eIF4G, which associates with Poly-A Binding Proteins bound to the poly-A tail. One class of repressors, eIF4E-BPs, binds to eIF4E on the same face that eIF4G binds, thus blocking the eIF4E-eIF4G interaction (Nakamura et al., 2004). Genes targeted by the eIF4E-BPs contain sequence specific sequences recognized by RNA binding proteins that complex with the eIF4E-BPs. Recent work has identified a variation on this mechanism in which an eIF4E homologous protein binds to the cap using the conserved cap-binding motif of eIF4E and this competition for the cap inhibits cap-dependant translation (Cho et al., 2005).
The binding of repressors to target sequences is the most common form of translational regulation found in eukaryotes. There are, however, several cases in which structural conformation of the sequence is the basis for the mechanism of repression. In these cases both the primary sequence and the secondary structure of the TCE is strictly conserved in order to maintain function (Crucs et al., 2000). The TCE of the *Drosophila* Nanos1 is located in the 3’UTR and contains two sequences that regulate the translation of the transcripts. The TCE was found to form a structure containing two hairpins, each of which binds a separate repressor. These stem-loops act independently to repress translation in the embryonic somatic cells and in the oocyte (Forrest et al., 2004, Kalifa et al., 2006). A second Nanos homolog, the *C. elegans* Nos-2 protein, is also translationally regulated by two independent stem-loops in the 3’UTR (D’Agostino et al., 2006). The Iron-Response-Element (IRE) is a stem-loop in the 5’UTR of the ferritin gene and other genes that regulate iron homeostasis (Theil and Eisenstein, 2000). The IRE is unique in eukaryotes because the stem-loop in the 5’ UTR does not inhibit initiation by interfering with the formation of the initiation complex, but rather it is recognized by the Iron Response Protein and together they sterically inhibit the scanning of the 48S initiation complex. Mechanisms that incorporate structural elements in RNA are rare in eukaryotes, and mechanisms based solely on structural elements without the need for a repressor have never been described. This is not true for prokaryotes however, where structural mechanisms of translational regulation are quite common. Riboswitches are a class of structural features in prokaryotic transcripts that can change their conformations by binding small-molecules or by changes in environmental temperature (Edwards et al., 2007). These conformational changes have been shown to cause rho-independent
transcription termination, activate self-cleavage, or obscure the Shine-Delgarno sequence repressing translation initiation (Gilbert and Batey, 2006; Edwards et al., 2007; Chowdhury et al., 2006).

It is with these mechanisms of translational regulation in mind that I approached the translational repression of Xnos1. Xnos1 is a Xenopus homolog of Drosophila Nanos1 and is expressed in many species of metazoans as a component of the germ plasm. The germ plasm is the germ cell determinant for many species and is a dense aggregate of endoplasmic reticulum, ribosomes, mitochondria, and germinal granules (Rongo and Lehmann, 1996; Draper et al., 2007; Chang et al., 2006). Xnos1 was identified in a screen for RNAs associated with the vegetal cortex of Xenopus oocytes (Mosquera et al. 1993). Xnos1, formerly called Xcat2, is transcribed during the early stages of oogenesis, localized to the mitochondrial cloud (Balbiani body), and packaged into germinal granules (Foristall et al. 1995; Zhou and King 1996; Kloc et al., 2002). Xnos1 is translationally repressed throughout oogenesis, during maturation events, and is not translated until early cleavage stages (MacArthur et al., 1999).

The proper regulation of Xnos1 expression is critical to normal development. Xnos1 and its Nanos homologs have been shown to be potent repressors of translation (Curtis et al., 1997; Kadyrova et al., 2007) and have been implicated in transcriptional regulation as well (Deshpande et al., 2005). The robust and overlapping translational repression of Nanos transcripts observed in many organisms makes sense when viewed in the context of germ cell specification vs. somatic cell specification. Early determination of embryonic cell fates is achieved by inheritance of morphogens during asymmetric
divisions of the cytoplasm and localized translation (Clements and Woodland, 2003; Stauber et al., 1999). The presence of these potent determinants in the cytoplasm that Primodial Germ Cells (PGCs) inherit must require a system of translational and transcriptional repression in order to retain the totipotency required for germ-cell fate. While this repressive activity of Nanos is required for PGCs to maintain their identity in the presence of somatic determinants, the somatic cells must also require that Nanos function be restricted to the germ-line to allow for somatic cell determination (Hayashi et al., 2004).

In this thesis I present a model for the translational repression of Xnos1. I propose that the translational regulation of Xnos1 is due solely to a structural motif in the first 75nt of the ORF that is predicted to form two stem-loops, Stem-loop A and Stem-loop B. Stem-loop A is a short stem-loop composed of 6 nucleotides of the 5’ UTR and the first 5 nucleotides of the ORF, while Stem-loop B forms 2 nucleotides down stream of Stem-loop A and is 64 nucleotides in length. I hypothesize that these predicted stem-loops work cooperatively to sterically prevent loading of the initiation codon into the P-site of the 48S initiation complex and thereby inhibit translation of Xnos1 transcripts. Such a structural mechanism for repression is completely novel for a eukaryotic RNA.
Chapter 2: Results

Translational repression of Xnos1 in oocyte extracts is not dependant on UTRs

Xnos1 is one of the maternal genes translationally repressed in the Xenopus oocyte (Macarthur et al., 1999). This repression was also active in-vitro using Xenopus oocyte extracts. Previous expression studies had revealed that Xnos1 transcripts are translated poorly when microinjected into oocytes, but not when translated in vitro using reticulocyte lysates. Repression of maternal transcripts is commonly regulated through translational control elements (TCEs) in the 3’UTR that serve as binding sites for proteins or small RNAs. Substitution of the Xnos1 3’UTR with the 3’UTR of β-globin failed to relieve repression (MacArthur et al., 1999). To identify which sequences constituted the Xnos1 TCE, a set of constructs were made in which the UTRs of the Xenopus β-globin gene replaced those of Xnos1 and these transcripts were tested for their translation ability in Stage VI oocyte extracts. β-globin serves as a useful control because both β-globin and Xnos1 have the same number of methionines, their UTRs are similar in length, and they are both similar in their apparent molecular weights. I first asked whether the Xnos1 UTRs were sufficient to repress translation of the β-globin open reading frame (ORF) in oocyte extracts. These hybrid constructs translated equally well as the β-globin RNA itself (Fig. 1, compare lanes 1 with 2 and 3). Similarly, substitution of the Xnos1 UTRs with those of β-globin UTRs failed to relieve repression of the Xnos1 ORF (Fig. 1, compare lanes 5-7 with 4). From these experiments, I conclude that the TCE of Xnos1 is not found within the UTRs. The failure of the Xnos1 UTRs to repress
translation of the β-globin ORF strongly suggested that the TCE lies within the Xnos1 ORF itself.

**Denaturing partially relieves repression of Xnos1 in oocyte extracts.**

The observation that the repression of Xnos1 transcripts in oocytes was not due to sequences in the 5’ or 3’ UTRs led us to look at the structure of the transcript using the MFOLD RNA structure prediction software (Walter et al., 1994, Zuker 2003). The MFOLD software computes the most probable folds in an RNA sequence based on lowest free energies of the folds and returns 25-35 separate predictions. There were 35 structures returned for Xnos1, with free energies from –348 kCal/mol to –337 kCal/mol. Due to the small differences in free energy the transcripts are most likely found in a distribution of all of these possible structures. In the lowest free energy structure returned, there are two stem-loops found within the first 90 nucleotides of the Xnos1 transcript (Fig. 2). These stem-loops, Stem-loop A and Stem-loop B, were of interest because: 1) Stem-loop A and B are found in the structures computed for *Xenopus borealis* and *laevis* Xnos1, and 2) Stem-loop A contains the AUG codon and suggests that the structure might inhibit initiation events. Stem-loop A, 11 nucleotides in length, was found in 20/35 of the structures predicted by the software. It contains the AUG initiation codon and 6 nucleotides of the 5’UTR. Stem-loop B is found in 16 of 20 structures that contained Stem-loop A and Stem-loop B is found only once without the presence of Stem-loop A suggesting that Stem-loop A is important in the stability of Stem-loop B.
I chose Stem-loop A for mutational analysis because of its correlation to Stem-loop B and because it contained the initiation codon. In order to eliminate Stem-loop A, the UCC nucleotides of the 5’ UTR in the stem were changed to AGG (Fig. 3A). The denatured constructs were tested for translation in both oocyte extract (not shown) and reticulocyte lysate (Fig. 3B). The AGG mutation did not translate at higher levels than wild-type Xnos1 in oocyte extract (not shown) or in reticulocyte lysate when the transcripts were denatured (Fig. 3B). I was expecting to see some relief of repression in the mutant, so this result was unexpected. It was likely that the small size of Stem-loop A was disrupted by the denaturing step prior to translation and therefore both the mutant and wt transcripts lacked Stem-loop A when denatured. I eliminated the denaturing step to assess the effect of the substitution mutation on translation of native transcripts. The native Xnos1<sub>AGG</sub> transcripts translated at almost 4 times the level of native Xnos1 transcripts suggesting that the denaturing step partially relieved repression. The difference seen in translation levels in native or denatured states was also observed when Xnos1 translation was compared to β-globin. β-globin was translated at a 4 fold higher level than Xnos1 when the transcripts were denatured and 10 fold when the transcripts were native. While a small increase in translation was observed for the Xnos1<sub>AGG</sub> construct, it is clear that Stem-loop A is not sufficient for Xnos1 repression. I conclude from these results that Stem-loop A only has a minor role in the translational repression of Xnos1 and that the effects of denaturing shows that structure does have a role in the mechanism for Xnos1 translational repression.
5’ but not 3’ ORF tag fusions relieve Xnos1 repression in vitro.

In other functional studies of Xnos1, I noticed that epitope tags cloned in frame 5’ to the Xnos1 ORF relieved repression and allowed translation of Xnos1 transcripts. I hypothesized that the 5’ tags might be disrupting some structure, such as the predicted Stem-loop B structure, preventing a repressor from binding. As a first step in discriminating between these two possibilities, I asked if the context of an engineered Myc-tag affects translation by placing it at the C-terminal end of the ORF. Myc-tagged Xnos1 constructs were generated by inserting the Xnos1 5’UTR and ORF either upstream (Xnos1-Myc) or downstream (Myc-Xnos1) of the 6x-Myc epitope tag in the pCS2-MT plasmid. Either placement of the Myc-tag was translated in reticulocyte lysate (RL) but only the transcripts with the Myc tag 5’ of the Xnos1 ORF were translated in oocyte extract (OE) (Fig. 4A, compare RL to OE). The translational repression of Xnos1-Myc but not Myc-Xnos1 is recapitulated in wheat germ extract (WG) (Fig. 4A). Fusing the Myc-tag downstream, but not upstream, of the Xnos1 ORF does not activate translation in oocyte or wheat germ extracts. The context dependent relief of repression seen in the oocyte and wheat germ extracts is inconsistent with the TCE acting as a sequence bound by a repressor, since such a trans-acting repressor would most likely be able to bind to the sequence and repress translation regardless of the location of the TCE (Elizabeth Gavis, personal communication).

Competition does not relieve Xnos1 translational repression.

If a translational repressor bound to Xnos1 RNA were required for repression, I would expect that excess Xnos1 RNA could compete out the repressor and relieve
repression. To test this, synthesized uncapped Xnos1 transcript was added in increasing amounts to oocyte extracts that contained a fixed amount of capped Xnos1 RNA. I used full-length Xnos1 RNA as the competitor and made the competitor transcripts uncapped in order to prevent translation of the competitor RNA. Because uncapped RNAs are inherently unstable, I first determined how long these transcripts would persist in oocyte extracts. After an hour incubation in oocyte extract, 28% of the initial 1 ug of radiolabeled transcript remained, 22% after two hours (Fig. 5A). This is in contrast to uncapped β-globin where after one hour, 70% of the radiolabeled message remained and 43% remained after 2 hours. I tested the ability of these uncapped transcripts to compete for the hypothetical repressor and subsequently allow the translation of the capped Xnos1 RNA. Increasing amounts of competitor transcripts, from 100 ng to 2 ug, were added to the extracts. The endogenous level of Xnos1 is estimated to be approximately 5 pg (MacArthur et al., 1999). Therefore, 2 ug corresponds to a full 400,000-fold excess over the endogenous message. Even with the decline in uncapped Xnos1 transcripts during the course of the assay, after 2 hours 22% of transcripts remained, or approximately an 80,000 fold excess over the 5 pg of endogenous Xnos1 transcript. Surprisingly, no differences in Xnos1 expression were detected at any dose of competition (Fig. 5B). Taken together, my results show that the Xnos1 TCE does not compete for a repressor and indicate that Xnos1 likely requires an activating factor(s) for its translation rather than the removal of a repressor.
Injected Xnos1-Myc transcripts are translated in vivo by 16-cell embryos.

In order to determine when such an activator might be present in vivo, Xnos1-Myc was injected into 1-cell embryos and embryos were collected at 16-cell and blastula stages. Xnos1-myc protein was detected after immunoprecipitation and western blotting with c-Myc antibody. Both Myc-Xnos1 and Xnos1-Myc protein fusions were detected at the 16-cell stage and in St. 7 embryos (Fig. 6). The Myc-Xnos1 appeared to translate at a higher level than Xnos1-Myc and expression of either one was significantly decreased by St. 7. The decrease in expression over time is most likely due to degradation of the message or protein product. The translation of Xnos1-Myc appears to mimic the behavior of endogenous Xnos1, that is, not translated in oocytes or oocyte extract, wheat germ extract, but translated very early in development. Recent work in our lab with a specific antibody against Xnos1 shows that endogenous Xnos1 may be detected early in cleavage stages. Interestingly, St. 10 embryo extract translates β-globin and Myc-Xnos1, but not Xnos1 or Xnos1-Myc transcripts (data not shown). One reason St. 10 embryo extracts may not translate Xnos1 or Xnos1-Myc RNA is that by this stage, the putative activating factor is degraded or diluted out. Taken together, these findings are consistent with the existence of an activating factor for Xnos1 RNA. Whether a regulatory factor(s) responsible for Xnos1 translation is specific for the germ plasm or not remains to be determined.

Xnos1-Myc does not form detectable 48S initiation complexes.

The context dependence of the Myc-tagged constructs together with the effects of denaturation on the translation of Xnos1, strongly suggested that structural inhibition may
play a key role in repressing Xnos1 translation. Toe-printing assays on eukaryotic ribosomes have established that the RNA binding cleft of a 48S complex is 30 nucleotides (Kozak, 1993). As described in Fig 2, Stem-loop B is predicted to form within 2 nucleotides of the predicted Stem-loop A, which contains the initiation codon. I hypothesized that Stem-loop B prevents translation by preventing the 48S complex from correctly positioning the initiation codon within the P site. If this were true, I would expect that Xnos1-Myc transcripts would not be able to bind the 43S initiation complex or form a competent 48S complex. To this end I performed sucrose gradient analysis on oocyte extracts containing $[^{35}\text{S}]$-UTP labeled Xnos1-Myc or Myc-Xnos1 transcripts and 500 ug/ml cycloheximide. Cycloheximide freezes translating ribosomes so I included it in my translation mix to increase the pool of radiolabeled transcripts in the 80S peak if any competent initiation events occurred. I did not see any detectable binding of the Xnos1-Myc construct to the 43S complex (Fig. 7A) while the Myc-Xnos1 transcripts exhibit weak binding (Fig. 7B). There is a slight shoulder to the Myc-Xnos1 CPM curve right at the 40S peak that I believe shows this weak binding in 3/3 trials. Unfortunately, these gradient experiments are not conclusive. One problem was that the unbound radiolabeled transcripts at the top of the gradient extend far enough into the gradient to partially overlap with the 43S peak seen in the 260nm absorbance plot. This overlap causes the shoulder in Myc-Xnos1 counts to be less definitive than it could be. An additional complication was that the *Xenopus* oocyte ribosomes require the presence of EDTA to disassociate into 43S and 60S subunits. The EDTA chelates Mg$^{2+}$ that the ribosomes require to maintain the proper RNA structures for association. The ribosomal
subunits also require Mg\(^{2+}\) in order to maintain their individual structures so the 43S subunit may not be able to bind RNA properly under the conditions tested.

I next tested the ability of 43S ribosomal subunits to bind the radiolabeled transcripts in the presence of GMP-PNP, a non-hydrolysable GTP analog. The recruitment of the 60S subunit is dependant on GTP hydrolysis and so including the GMP-PNP in the translation mix prevents 60S subunit recruitment and increases the amount of 48S complex bound to mRNA even in the presence of Mg\(^{2+}\). Sucrose gradient analysis with GMP-PNP shows that there was some binding of the Myc-Xnos1 transcript to the 43S subunit compared to the binding of Myc-Xnos1 to the 43S subunit with cycloheximide (compare Fig. 8A and B). The free transcripts are still overlapping enough with the 43S subunit to make any signal less obvious. Optimization of the sucrose gradients will be required to allow more separation.

More than 3mM EDTA is required to separate ribosomal subunits.

The fact that EDTA is required to dissociate the ribosome into its subunits is problematic because Mg\(^{2+}\) is required for subunit integrity as well as ribosome assembly. In order to determine if it is possible to chelate enough Mg\(^{2+}\) to dissociate ribosomes but preserve subunit structure I performed sucrose gradient analysis using several concentrations of EDTA.

Figure 9 shows that 1 mM EDTA was insufficient to dissociate the ribosomes but 5 mM EDTA was able to dissociate the ribosomal subunits. The translation mix is supplemented with 3 mM Mg\(^{2+}\) acetate so sucrose gradient buffer containing 2.5 mM and
3 mM EDTA were tried hoping that this would yield a less severe chelation (Figure 10). Neither concentration of EDTA was able to separate the ribosomes into subunits.

**Isolated 40S subunits fail to bind the Myc-Xnos1 transcripts.**

The requirement of the 43S subunit for Mg$^{2+}$ ions to bind RNA presents an obvious problem if the presence of Mg$^{2+}$ causes subunits to form the 80S ribosome. Isolation of 43S subunits in the presence of EDTA and then resuspension of the subunits in a buffer containing Mg$^{2+}$ allows for a more direct binding assay as well as removal of most of the free RNA found in the oocyte extracts. To isolate ribosomes, oocyte extracts were first spun through a sucrose cushion made up in either high salt or low salt conditions (Figure 11). High Salt sucrose cushions cause the ribosome to lose its associated translation factors and accessory proteins and the low salt cushion preserves these intermolecular interactions. The 43S subunits were then incubated with the radiolabeled transcripts and then centrifuged in a sucrose gradient. The isolated 43S subunits did not bind the Myc-Xnos1 despite the fact that these transcripts are readily translated in oocyte extract (data not shown). Future work to determine ribosome binding should use the GMP-PNP because the GTP analog prevents 60S recruitment to the 48S complex without the use of EDTA and purification steps that may also remove key ribosomal proteins.
Chapter 3: Figures

Figure 1. TCE of Xnos1 is located in the ORF.

A. Schematic of the constructs tested showing UTR substitutions. X represents Xnos1 sequences and B represents β-globin sequences. Uppercase represents the ORF and the UTRs are in lowercase.

B. In vitro translation analysis of UTR substitution constructs in oocyte extracts. Arrow heads mark the position of Xnos1.
Figure 2. A conserved secondary structure predicted in the first 75nt of the Xnos1 ORF. 
A. The lowest free energy structure of 35 structures for full length Xnos1 predicted by 
the MFOLD program, version 3.2 (Zuker, 2003, Walter et al., 1994). B. Close up view 
of the conserved stem-loops from the inset in A. The black circle represents the cap and 
the green highlight marks the initiation codon. C. Lowest free energy structure for full-
length X. borealis Xnos1 predicted by the MFOLD program, version 3.2 (Zuker, 2003, 
Walter et al., 1994). D. Close up view of the conserved stem-loops from the inset in C.
Figure 3. Denaturing relieves Xnos1 repression in oocyte extract.
A. Schematic of Stem Loop A and the substitutions in the Xnos1_{AGG} mutant. B. Control gel showing \[^{35}\text{S}^\]-Methionine labeled proteins from in vitro translation of my constructs in reticulocyte lysate. C. Comparison of the translation of \(\beta\)-globin, Xnos1, and Xnos1_{AGG} transcripts with or without denaturing. 1 ug of each transcript was translated in an equal volume of reticulocyte lysate or oocyte extract.
Figure 4. Context dependence of Myc-Xnos1 fusion constructs.
A. *in-vitro* translation assays labeled with $[^{35}S]$-Methionine. 100 ng of each transcript was added to 9 ul of translation extract. OE (oocyte extract); WG (wheat germ extract); RL (reticulocyte lysate). Closed arrowhead marks the migration of Myc-Xnos1 fusions, open arrowhead marks the migration of Xnos1 and $\beta$-globin. B. Schematic of the orientation of the Myc-tagged constructs. C. Summary of the results of the translation analysis in A.
Figure 5. Competition does not relieve Xnos1 repression.
A. Stability of the uncapped competitors. 1 ug of $[^{35}\text{S}]$-UTP labeled uncapped transcripts were incubated in oocyte extract translation mix for 6 hrs. Samples were taken at time points shown and analyzed on a Urea-Page gel. B. Histogram showing results of the competition experiment. 25 ng of capped Xnos1 or β-globin transcripts were translated for 90 minutes at room temperature in oocyte extract containing $[^{35}\text{S}]$-Methionine. Un-capped Xnos1 transcripts were added to the translation mix in increasing amounts as shown. Xnos1 and β-globin contain the same number of methionines and are approximately the same length. 100 ng of Xnos1 was translated in labeled reticulocyte extract to provide a positive control.
Early stage embryos translate Xnos1-Myc

Figure 6. Early stage embryos translate Xnos1-Myc transcripts. 1 ng of capped transcripts was injected into 1-cell stage embryos. Embryos were collected at the indicated stages and 5 embryos were lysed for each immunoprecipitation. Blots were probed with α-Myc monoclonal antibodies (Invitrogen, 13-2500) and stained with BM purple.
Figure 7. Xnos1-Myc does not form measurable 48S complexes subunit.
100 ng of [35S]-UTP labeled transcripts were added to 100 ul of oocyte extract translation mix and incubated for 45 minutes. Translation was stopped with 100ul of ice-cold sucrose gradient buffer containing 500 µg/ml cycloheximide and 6mM EDTA. This solution was layered over a 14 ml 10-30% linear sucrose gradient and centrifuged for 5 hrs at 33k RPM in an SW40 rotor. 0.5 ml fractions were collected from the bottom, 200 ul was taken for A260 nm readings and 100 ul was dried in glass vials and read in a scintillation counter after overnight incubation in the dark.
Figure 8. GMP-PNP causes increased binding of Myc-Xnos1 to 40S subunit. 12.5-30\% Gradient demonstrating GMP-PNP prevents subunit assembly and allows for binding of Myc-Xnos1 to 43S complex. 100 ng of \(^{35}\)S-UTP labeled transcripts were added to 100 ul of oocyte extract translation mix and incubated for 45 minutes. Translation was stopped with 100 ul of ice-cold sucrose gradient buffer containing 500 ug/ml cycloheximide or 2 mM GMP-PNP. This solution was layered over the 14 ml linear sucrose gradients and centrifuged for 5 hrs at 33k RPM in an SW40 rotor. 0.5 ml fractions were collected from the bottom, 200 ul was taken for A260 nm spectrophotometry and 100 ul was dried in scintilation vials and read after incubation overnight in the dark.
Figure 9. EDTA is required to separate ribosome into subunits.
12.5-30% sucrose gradient demonstrating that EDTA is required to separate ribosome into subunits. 100 ng of transcripts were added to 100 ul of oocyte extract translation mix and incubated for 45 minutes. Translation was stopped with 100ul of ice-cold sucrose gradient buffer containing 500 ug/ml cycloheximide and 1 mM or 5 mM EDTA. This solution was layered over the 14 ml linear sucrose gradients and centrifuged for 5 hrs at 33k RPM in an SW40 rotor. 0.5 ml fractions were collected from the bottom, 200 ul was taken for A260 nm spectrophotometry.
Figure 10. EDTA in excess of Mg$^{2+}$ is required to separate ribosome into subunits. 12.5-30% sucrose gradient demonstrating that EDTA is required to separate ribosome into subunits. 100 ng of transcripts were added to 100 ul of oocyte extract translation mix and incubated for 45 minutes. Translation was stopped with 100 ul of ice-cold sucrose gradient buffer containing 500 ug/ml cycloheximide and 2.5 mM or 3 mM EDTA. This solution was layered over the 14 ml linear sucrose gradients and centrifuged for 5 hrs at 33k RPM in an SW40 rotor. 0.5 ml fractions were collected from the bottom, 200 ul was taken for A260nm spectrophotometry.
A. Low-Salt Washed Ribosome Isolation

B. High-Salt Washed Ribosome Isolation
Figure 11. Ribosome subunit isolation.
A. Oocyte homogenate was centrifuged through a low salt sucrose cushion with EDTA (LSE) or without EDTA (LSM), and centrifuged for 5 hrs at 48k RPMs in a 60Ti rotor to obtain a ribosome pellet. The ribosome pellet was then resuspended in RBS containing Mg\(^{2+}\) or EDTA and rocked overnight at 4\(^\circ\)C. This solution was then layered over a 14 ml 10-30% sucrose gradient in either high-salt or low-salt conditions and centrifuged for 19 hrs at 23k RPM in an SW28 rotor. 1 ml fractions were collected from the bottom and A\(_{260}\)nm was determined by reading 200 ul aliquots on a microplate spectrophotometer.
B. Oocyte homogenate was centrifuged through a high salt sucrose solution with EDTA (HSE) or without EDTA (HSM) as described above.
Figure 12. Model for Xnos1 translational repression.
A. Stem-loops A and B, located in the first 75 nt of the mRNA and a short 15nt 5’UTR prevent efficient loading of the P-site on the 43S complex. The green shading represents the initiation codon; the black closed circle represents the 5’ cap.
B. Melting of Stem-loop A and B by denaturing transcripts, Stem-loop A substitutions, activation by helicases or stochastic fluctuations in structure allows loading of the P-site and formation of the 48S initiation complex. The possible helicase is represented by the purple oval.
C. Insertion of coding sequence upstream of Xnos1 would permit 43S complex binding and translation of Xnos1 because ribosomes can move through regions of structure once they are in the elongation phase.
Chapter 4: Discussion

My data presented here are most consistent with a model in which the 43S complex is prevented from initiating translation on Xnos1 transcripts by structural elements predicted in the first 75nt of the ORF (Fig. 12A). These structural elements repress translation by sterically inhibiting the loading of the AUG start codon into the P-site of the 48S complex. This model predicts that removal of these structures by a helicase, mutation, or denaturation would allow for weak 43S complex binding events to allow 48S complex formation and subsequent recruitment of the 60S ribosome and translation (Fig 12B). Denaturing Xnos1 transcripts and the Stem-loop A substitution mutant both caused a partial relief of repression in my translation assays. I believe this is due to the increased accessibility of the AUG to the P-site by removal of Stem-loop A. The context dependant translation of Xnos1-Myc constructs also supports this hypothesis. When Xnos1 is inserted upstream of Myc the 48S complex is not formed because of the presence of the predicted stem-loops. When Myc is upstream of Xnos1, however, the 48S complex forms normally on the Myc ORF, recruits the 60S subunit, and the 80S translating ribosome is able to melt away any secondary structure in the Xnos1 ORF resulting in high levels of expression (Fig. 12C).

This work began with the observation that Xnos1 transcripts are poorly translated or not translated at all when injected into oocytes. Substitution of the Xnos1 3’UTR with the 3’ UTR of β-globin had no effect; Xnos1 remained repressed. The failure of Xnos1 ORF to translate despite replacement of the 5’ and 3’ UTRs demonstrates that the TCE lies within the ORF of Xnos1 (MacArthur et al., 1999, and this work). I tested the effects of Stem-loop A mutations and mRNA denaturation on translation and found that
both worked together to partially relieve repression. Two lines of evidence argue against a requirement for a translational repressor in Xnos1 repression: 1) The failure to compete out a repressor with excess Xnos1 transcripts; 2) The context dependence of repression observed when a Myc tag is inserted upstream or downstream of Xnos1. The Xnos1-Myc constructs also failed to measurably bind the 43S complex in sucrose gradient analyses. These results are all consistent with a model in which a structural element in the 5’ end of the Xnos1 ORF prevents the 43S initiation complex from loading the initiation codon into the P-site of the 48S complex and recruiting the 60S subunit.

My P-site loading model, (Fig 12) depends on two critical assumptions: 1) preventing P-site loading of the initiation codon effectively inhibits translation, and 2) the predicted stem-loops have enough stability to prevent the initiation complex from melting them and thus prevent loading the start codon into the P-site. Interestingly, published work on 5’UTR length and translation in *S. cerivisae* demonstrated that a *cat* reporter with 22nt of 5’UTR translated at 20% the rate of the same *cat* reporter with a 77nt 5’UTR (Sagliocco et al., 1993). This is especially significant in light of toe-printing and RNAse protection assays showing the RNA binding cleft of the ribosome to be approximately 30nt in length (Pestova et al 2001, Kozak 1998). Based on these observations, the short 5’UTR and inhibitory structures proximal to the initiation codon in Xnos1 could sterically prevent P-site loading and provide for a robust translational repression.

Stem-loops with a free energy ≥ -50 kCal/mol inhibit ribosomal scanning in COS cells (Kozak 1986). 5’UTR stem-loops of -20-30 kCal/mol almost completely inhibit *cat* gene expression in *S. cerivesae* (Sagliocco et al., 1993). Stem-Loop B in *Xenopus laevis* is predicted to have a free energy of –56 kCal/mol and Stem-loop A has a predicted free
energy of –17 kCal/mol. These predicted free energies and the results from Kozak (1986) and Sagliocco et al., (1993) are consistent with my hypothesis that Stem-loop A and Stem-loop B, together with the short 15nt 5’UTR, prevent loading of the initiation codon into the P-site of the 48S initiation complex.

According to my model, insertion of coding sequence between Stem-loop A, which contains the AUG initiation codon, and Stem-loop B would allow the scanning 48S initiation complex to load the initiation codon into the P-site and should allow for translation of Xnos1 in oocyte and wheat germ extracts. New constructs incorporating varying lengths of coding sequence between the Stem-loop A and Stem-loop B could be tested *in vitro* and *in vivo* to test the inhibition of P-site loading mechanism of repression.

To fully describe the mechanism of the Xnos1 TCE requires finding the germ-line activator of Xnos1 translation and creating constructs that would test my P-site loading hypothesis. The immunoprecipitation experiment against the Xnos1-Myc fusion protein demonstrated that embryos are able to translate Xnos1 ORF as early as the 16-cell stage and recent work in the lab with an α-Xnos1 polyclonal may have detected Xnos1 protein as early as the 4-cell stage. The 4-cell stage should contain an activator of Xnos1 translation and fractionation of 4-cell extract could identify the fraction required to relieve repression.

The model presented is appealing because it offers a simple explanation for how somatic cells prevent translation of any un-localized Xnos1 mRNA. Nanos family members are potent repressors of somatic cell fates by functioning as repressors of both transcription and translation (Curtis et al., 1997; Kadyrova et al., 2007; Deshpande et al., 2005). The need to restrict Nanos translation to the germ line is clear. Structural
inhibition of translation would provide a robust repression independent of the proper localization of the message or the proper expression of a repressor. The structural inhibition of translation is novel in eukaryotes but has been shown repeatedly in the regulation of prokaryotic mRNAs (Gilbert and Batey, 2006; Edwards et al., 2007; Chowdhury et al., 2006).

The possibility that Xnos1 is regulated like a prokaryotic message is intriguing because it is consistent with work published by Saturo Kobayashi that suggests that mitochondrial ribosomes are translating a message in the germ plasm of *Xenopus* and *Drosophila* embryos. This hypothesis, although controversial (Kloc et al., 2001), has been examined by a variety of approaches including *in situ* hybridization, knockdown, rescue, and ultra-structural studies. mtrRNAs are present in the germ plasm outside the mitochondria during the first stage of *Drosophila* embryogenesis up to pole cell formation and the 4-cell to blastula stages in *Xenopus* (Kobayashi et al., 1998; Kashikawa et al., 2001). Knockdown of cytoplasmic mtrRNA prevents formation of the germline and this effect can be rescued by injection of *in vitro* transcribed mtrRNAs (Kobayashi et al., 1998; Amikura et al., 2001). At the ultrastructural level, polysomes visualized by EM on the surface of germinal granules and polar granules correspond in size to mitochondrial ribosomes instead of eukaryotic ribosomes (Amikura et al., 2001). The possibility that Xnos1 is a mRNA translated by mitochondrial ribosomes in the cytoplasm is intriguing and I predict Xnos1 translation *in vivo* would be sensitive to chloramphenicol, an inhibitor of prokaryotic and mitochondrial translation but not eukaryotic translation.
The results presented here support the hypothesis that Xnos1 RNAs are translationally repressed by a novel mechanism in eukaryotes. My data argues against the requirement for a trans-acting repressor protein in the repression and instead argues for a purely structural inhibition of translation. The model proposes two stem-loops that inhibit loading of the initiation codon into the P-site of the 48S subunit and prevents recruitment of the 60S subunit and subsequent translation. My current reasoning is that due to the potent transcriptional and translational inhibition exhibited by Nanos family members it follows that an equally robust repression and activation of Xnos1 translation would be required. The most robust translational repression would be intrinsic to the transcript and independent of other factors. Future work will determine if the predicted structural features in the ORF do indeed exist and prevent Xnos1 from binding to the 43S complex.
Chapter 5: Materials and Methods

Plasmid Construction

Xnos1: Full Length Xnos1 cDNA was cloned into pSport1 vector as a SalI-NotI insert. The insert contains 15nt of 5’UTR, 384nt ORF, and 380nt 3’UTR. It is cut with HindIII and transcribed with T7 for sense or cut with EcoRI and transcribed with SP6 for anti-sense.

β-Globin: The β-globin transcripts were generated from the pSPXβM (Melton et al., 1984) cut with EcoRI and transcribed with SP6 for sense transcripts.

Xnos1_{AGG}: Xnos1 was amplified with a forward primer that replaced the TCC nucleotides with AGG at position 10-12 in the 5’UTR. The insert generated was cut with SalI and NotI and cloned back into a pSport1 vector.

Myc-Xnos1: Xnos1 coding region was amplified from the Xnos1 pSport1 vector, digested with EcoRI and XhoI and cloned into the pCS2-Myc vector.

Xnos1-Myc: Xnos1 was 5’UTR and ORF was amplified from the Xnos1 pSport1 vector, cut with BamHI and ClaI and cloned into the pCS2-Myc plasmid. All clones were verified by sequencing.

Isolation of Oocytes from adult Xenopus females

Xenopus laevis adult females were anesthetised for 20 minutes in 0.5% MS222 (Sigma A5040) in 0.1x MMR (0.1M NaCl, 2.0 mM KCl, 1mM MgSO4, 2 mM CaCl2, 5mM HEPES 7.4). One or both ovaries were removed and placed in a dish of OR2 (82.5 mM NaCl, 2.5 mM KCl, 1mM CaCl2, 1mM MgCl2, 1 mM Na2HPO4) buffer. The ovarian sacks are then opened with forceps to expose the oocytes and the pieces placed in a 50ml conical tube with 4500 units of collagenase type I (Worthington, CLS1 4197) in 30 mls
of sodium phosphate buffer (65.5 mM Na$_2$HPO$_4$, 26.7mM NaH$_2$PO$_4$). The tube was rocked until the oocytes were free from the theca. The oocytes were rinsed twice with 20mls of 2% BSA in OR2 followed by three rinses of 50ml of OR2. Stage IV-VI oocytes were sorted by gravity sedimentation during several rinses by inverting the 50ml tube and then pouring off the OR2 with the St. I-III oocytes that settle more slowly.

**Preparation of Extracts for in vitro translation**

Oocytes or embryos were washed three times in ice-cold CSF-XB buffer (17.11g sucrose, 50ml 20x salts (2 M KCl, 20 mM MgCl$_2$, 2 mM CaCl$_2$), 10ml 1M Hepes pH 7.3, 10ml 0.5 M EGTA pH7, 930ml DI water) and kept on ice. Oocytes and embryos were transferred to a 15 ml glass Corvex tube that had been chilled on ice. After removal of as much buffer as possible, the tube was spun in a Sorval HB4 rotor at 10,000 RPM for 10 minutes at 4°C. The aqueous phase between the pelleted debris and the lipid layer was transferred to a fresh Corvex tube and spun for an additional 10 minutes at 10,000 RPM at 4°C. The aqueous phase was then transferred to a new Corvex tube 600ul at a time. For each 600ul of lysate 100ul of 7x protease inhibitor (1 pellet of inhibitor in 1ml DI water, Roche) was added and mixed by vortexing. Aliquots of 35ul or 100ul were frozen in dry ice and stored in the -80 freezer.

**In-Vitro Transcription**

For capped transcripts, 300ng to 1ug of linearized plasmids were used in the SP6 or T7 mMessage Machine transcription kits from Ambion. For uncapped transcripts the SP6 or T7 MEGAscript kits were used. Template DNA was removed from the 20ul transcription reaction by DNAse treatment followed by addition of 1/10$^{th}$ volume of ammonium acetate and phenol/chloroform extraction to remove all the proteins. The RNA was
precipitated by addition of one volume of ice-cold isopropanol and put at -20°C for at least 30 min or overnight. RNA was pelleted by centrifugation at 14k rpm at 4°C for 30 minutes. The RNA pellet was then resuspended in 10ul of nuclease free water and concentration was determined by UV spectrophotometry. RNA was stored at a concentration of 1ug/ul and microinjection and aliquots for in vitro translation reactions were made at 100ng/ul or 25ng/ul.

**In vitro translation with oocyte or embryo extracts**

The translation mix was prepared from 35 ul of oocyte extract, 14 ul DI water, 4 ul CTX salts (833 mM KCl, 20 mM magnesium acetate, 16.7 mM DTT), 6.25 ul 200 mM creatine phosphate (Sigma, P7936), 1.25ul Creatine Kinase (stored as 20 mg/ml rabbit muscle creatine kinase (Sigma C3755) in 50% glycerol and 125 mM gly-gly (Sigma G11002)), 1.25ul 5mg/ml calf liver tRNA, 1ul amino acid mix –MET (Promega), 2 ul 100 mM DTT, 1ul RNasin (Ambion), 1ul of methionine or \[^{35}\text{S-Methionine}\] was used as indicated. Each translation reaction contained: 25 ng-1ug of RNA in 1ul and 9 ul of translation mix; and was incubated at room temperature for 2hrs. The reactions were stopped by addition of one volume of 2x SDS loading buffer and one volume of 1x SDS loading buffer and boiled for 10 minutes followed by cooling in ice bath for 5 minutes. Samples were used fresh or stored at –20°C.

**In vitro translation with reticulocyte lysate and wheat germ extract**

The translation mixes were made according to the manufacturers instructions and 100ng of each RNA was added. \[^{35}\text{S-Methionine}\] was used as a radiolabel where indicated.
Electrophoresis

12 or 15% Acrylimide gels were cast in 0.75mm Mini-Protean plates from BioRad. 10ul of sample or pre-stained protein ladder is added to each lane and run at 200V and constant amps for approximately 50 minutes or until the dye front reached the bottom of the gel to preserve low molecular weight proteins. Gels were then dehydrated in 20% methanol and 10% acetic acid to prevent cracking and dried on filter paper in the vacuum drier at 80° for 2hrs.

Autoradiography

Dried gels were exposed for 1 to 3 days and the bands were quantified using the ImageQuant software package.

Immunoprecipitation

1 ng of Myc-Xnos1 or Xnos1-Myc capped transcripts was injected into 1-cell Xenopus laevis embryos. Samples of 5 embryos per time point were collected and lysed in 100 ul of Immunoprecipitation Lysis Buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P40; 1 tablet Roche Complete protein inhibitor cocktail/50 ml). 50 ul of Protein G agarose beads (Roche 11 719 416 001) were added to the lysate and incubated overnight on a rocking platform at 4°C. The agarose beads were removed by centrifugation and the supernatant moved to a fresh tube. 1 ug of α-cMyc antibody (Invitrogen 13-2500) was added to the cleared lysate and the tubes were incubated for an hour on a rocking platform at 4°C. 50 ul of Protein G bead suspension was added and the tubes were incubated overnight on a rocking platform at 4°C. After the overnight incubation the agarose beads are collected by centrifugation and washed three times in 1 ml of Lysis Buffer, three times with 1 ml of Wash Buffer 2 (50 mM Tris-HCl, pH 7.5;
500 mM NaCl, 0.1% Nonidet P40) and washed one final time in Wash Buffer 3 (50 mM Tris-HCl, pH 7.5; 0.1% Nonidet P40). After the final wash the agarose beads were resuspended in 20 ul 1x SDS loading buffer and boiled for 5 minutes before the supernatant was moved to a fresh tube.

**Western Blotting**

Samples were electrophoresed as described above. After electrophoresis the gels are soaked in transfer buffer (25 mM Tris, 1.92 M Glycine, 0.1% SDS, 20% methanol) for 10 minutes along with 2 pieces of filter paper and one piece of nitrocellulose membrane. The blotting sandwich was made as follows: filter paper, gel, nitrocellulose, filter paper; and oriented with the gel towards the cathode (negative). The blots were transferred overnight at 30 mV. The following morning the blots were blocked with 5% non-fat skim milk in 1X PBS-T (1X PBS with 0.05% Tween-20) on a rocking platform at room temperature for 30 minutes. The blots were incubated in 2 mls of 1% non-fat skim milk with primary antibody (Invitrogen 13-2500) at a 1:1000 dilution for 60 minutes at room temperature on a rocking platform. The blots were rinsed in PBS-T twice for 15 minutes and then incubated in 1% non-fat skim milk with AP-conjugated secondary antibody at 1:5000 on a rocking platform for 30 minutes at room temperature. The blots were then washed three times in PBS-T and then placed in a square plastic dish and covered with BM-Purple staining solution (Roche 11 442 074 001) and incubated at 4°C until sufficient color has developed.

**Sucrose Gradient Experiments**

100ul of oocyte extract translation mix was mixed with 100ng of radiolabeled transcripts and incubated at room temperature for 20 minutes. The incubations are then stopped
with addition of cold sucrose gradient buffer, SGB: (24mM Hepes-KOH 7.5, 100mM K acetate, 0-3mM Mg acetate, 100mM DTT or 5mM 2ME, 500ug/ml cycloheximide or 2mM GMP-PNP) and layered onto linear sucrose gradients. The linear gradients were prepared by filling a 14 ml or 40 ml polyallomer tube (Beckman) 2/3rds full with a light sucrose solution (10-15% in SGB). A syringe with the plunger removed was then used to deliver a high sucrose solution (25-50% in SGB). When the high sucrose solution displaced the low sucrose solution to the half-way mark (using the aluminum die made by Beckman) the syringe was removed and the tube plugged and placed in the Gradient Master set at: Speed = 76, Angle = 21, Direction = up. The gradients were then centrifuged at 33k RPM for 4 or 5 hrs at 6°C for SW40 rotors and 23k RPM for 15 or 19 hrs at 6°C in SW60 rotor. 0.5ml or 1ml fractions were collected from the bottom using a peristaltic pump and the absorbance at 260nm is determined by taking 200 ul for spectrophotometry. 100 ul of each fraction was also dried in a scintillation vial followed by addition of 10 mls of scintillation fluid. Counts were read after storing over night in the dark.

**Ribosome Isolation**

1 ml of embryos or oocytes were homogenized in 3mls HRS buffer (10mM Hepes-KOH 7.5, 10mM MgCl$_2$, 50mM NH$_4$Cl, 5mM 2ME, 1 U/ml DnaseI, 1 U/ml RNasin) and placed on ice for 15 minutes. Homogenate was centrifuged for 30 minutes at 16k RPM in a Sorvall SS34 rotor or equivalent (30k RCF) at 4°C. The supernatant should be free of debris and lipid, if not, most of the aqueous phase was transferred to a new tube and centrifuged an additional 15 minutes. The homogenate was layered over a sucrose cushion made in a high salt (20% sucrose, 10mM Hepes-KOH 7.5, 10mM MgCl$_2$,
1MNH₄Cl, 5mM 2ME) or low salt buffer (40% sucrose, 10mM Hepes-KOH 7.5, 10mM MgCl₂, 50mM NH₄Cl, 5mM 2ME) and then centrifuged for 5hrs at 37k RPM in a 60Ti rotor (137k RCF). The ribosome pellet was a transparent orange gel at the bottom of the tube. The pellet was resuspended in Low Mg HRS (HRS with 0.1mM MgCl₂ and 1mM edta) to separate subunits and then rocked for 2hrs or overnight at 4ºC. The purified ribosome suspension was then centrifuged in a 15-35% linear sucrose gradient in Low Mg HRS buffer for 19 hrs at 21k RPM in an SW28 rotor. 1 ml fractions were collected from the bottom using a peristaltic pump and 50ul was taken to measure the A260nm. Fractions containing 40S subunits were pooled and then centrifuged through a 20% sucrose cushion in HRS buffer at 37k RPM in a 60Ti rotor at 4ºC. The subunit pellet was then resuspended in HRS buffer, frozen on dry ice, and stored at -80 ºC.

40S binding Assay

50-100 A260 units of purified 40S ribosomal subunits were incubated with 100ng of radiolabeled transcript for 15 minutes in RB buffer (10mM Hepes-KOH 7.5, 10mM MgCl₂, 125mM KCl, 5mM 2ME, 1 ul RNasin) in a total volume of 500ul. The mix was then layered onto a 15-35% linear sucrose gradient in HRS and centrifuged for 5hrs at 23k RPM in an SW28 rotor at 4ºC. 1 ml fractions were collected from the bottom and 200 ul was taken for A260 nm and 100 ul was dried in a scintillation vial and then mixed with 10 mls of scintillation fluid. Counts were determined after an overnight incubation in the dark.
References:


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