RELEVANCE OF UPSTREAM OPEN READING FRAMES IN eIF5 AND INOSITOL-3-PHOSPHATE SYNTHASE TRANSCRIPTS IN *NEUROSPORA CRASSA*

A Senior Scholars Thesis

by

CHRISTOPHER GENE BENNETT

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Majors: Biology
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ABSTRACT

Relevance of Upstream Open Reading Frames in eIF5 and Inositol-3-Phosphate Synthase Transcripts in *Neurospora crassa*. (April 2011)

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Understanding the mechanism of posttranscriptional gene control is of growing significance. Upstream open reading frames (uORFs) are of particular interest since they appear throughout the eukaryotic kingdom, from simple yeast to humans. These elements have the potential to regulate the translation of their associated open reading frame (ORF) when they are, themselves, translated. The focus of this project was to determine if the upstream open reading frames present in eIF5 and inositol-3-phosphate synthase transcripts in *Neurospora crassa* have regulatory activity. These have regulatory activity as determined through *in vitro* studies, using a luciferase assay, that measures the activity of an upstream open reading frame by placing it upstream of a luciferase reporter gene. The results of this assay were then compared with toeprinting data, performed by Cheng Wu in the lab, to confirm the results. As new information on

genetic mechanisms emerges, so does the understanding of processes common for all eukaryotic organisms.

ACKNOWLEDGMENTS

I would like to thank: Dr. Matthew Sachs for allowing me to work in his laboratory, Jiajie Wei for helping and mentoring on this project, Cheng Wu for his toeprinting date and everyone in the lab for their help. The Undergraduate Research Scholars provided funding for part of my research.

NOMENCLATURE

DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
eIF5	Eukaryotic Initiation Factor 5
I3PS	Inositol-3-Phosphate Synthase
ORF	Open reading Frame
p2	Plasmid 2 with I3PS uORF
p4	Plasmid 4 with eIF5 uORF 1
p6	Plasmid 6 with eIF5 uORF 2
PCR	Polymerase Chain Reaction
uAUG	Upstream AUG
uORF	Upstream Open Reading Frame
UTR	Untranslated Region

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CHAPTER I

INTRODUCTION

Gene regulation can occur at any step of the expression process, with some regulatory mechanisms better understood than others. Posttranscriptional regulation is one of the least understood mechanisms. It is of critical importance to know how these regulatory pathways are achieved and regulated if gene control is to be fully understood.

Upstream open reading frames (uORFs) can play a major role in translational regulation. uORFs are short sequences of nucleotides located in mRNA between the cap and the downstream gene in the area that is generally called the 5' untranslated region (UTR). uORFs typically range anywhere from two codons to 50 codons. Their start codons can vary from the normal AUG codon and consist of alternative start codons (Iacono et al., 2005). It is, then, conceivable that most uORFs with an uAUG start codon are stronger regulators than those with alternative start codons. Though uORFs are not the only mechanism of translational control, they hold great potential to control protein production (Morris and Geballe, 2000). These elements function when they are translated, by affecting ribosome reinitiation, slowing the ribosome or stalling the ribosome while elongating (Hood et al., 2009). The act of translating an uORF alone may down-regulate the downstream gene, since choosing the uORF start-codon can prevent the ribosome from reaching the downstream ORF (preemptive initiation).

This thesis follows the style of *Cell*.

Alternatively, the nascent peptides made by the uORFs can mediate ribosome stalling alone or in response to a small molecule that subsequently leads to down regulation of the gene product by preventing other ribosomes from scanning (Fang et al., 2004; Gong and Yanofsky, 2004). Regulatory uORFs have been found throughout the eukaryotic kingdom (Crowe et al., 2006). Certain human diseases have been attributed to the inactivity of uORFs (Wethmar et al., 2010). By understanding how these elements work and their impact on gene expression we can expand our knowledge of how genes are controlled and how to potentially alter expression to combat genetic diseases.

This research focuses on the uORFs in eIF5 and inositol-3-phosphate synthase (I3PS) genes in *Neurospora crassa*. These genes are vital to the health of the cell and are tightly regulated, thus their mechanisms of control need to be better understood. I3PS is the first enzyme in the inositol biosynthesis pathway, an important secondary messenger in eukaryotes that is used extensively in multicellular organisms. Inositol is a vital component to living cells as it directs membrane biogenesis and cell growth, and when cells become inositol starved they grow slower and eventually die (Fischbach et al., 2006). There is, also, some evidence that a decreased level of inositol in humans is linked to depression which can be treated with inositol supplements (Levine et al., 1995). eIF5 is a translation initiation factor used in eukaryotes. It binds with other initiation factors to form a complex that attaches the small ribosome subunit to eIF2-Met-tRNA complex to create the 43S preinitiation complex. Some studies suggest that

eIF5 remains associated with the ribosome during elongation and is important for processivity (Park et al., 2009; Valašek et al., 2003). An imbalance in eIF5 could be maladaptive to the cell. If not enough is made the cell may not be able to produce enough proteins in response to survive and reproduce, however, too much eIF5 may be a waste of energy and distort cellular machinery. eIF5 has two possible uORF's in its 5' UTR while I3PS has only one. Unpublished data (not shown) from our collaborator Ivaylo Ivanov (University of Utah) suggest that these elements function in translational control of there respective gene products. Figure 1 shows the mRNA sequence of these uORFs. Both of eIF5's uORFs have AUG start codons while I3PS's uORF does not. Therefore, it can be hypothesized that the eIF5 uORFs will have stronger regulatory activity than I3PS since the initiation codon is stronger and more easily recognized.

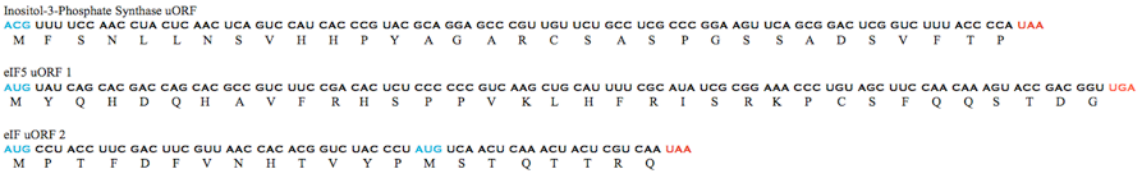

Figure 1. uORF mRNA sequences
The uORF sequences with initiation codons in blue and termination codons in red. The translated sequence is shown below the mRNA sequence.

The objective was to find out whether or not each of these uORFs are functional regulators and to determine the magnitude of the regulatory effects. *In vitro* luciferase assays were used to assess the activity of these elements. Luciferase is an enzyme from firefly that produces luminescence with the appropriate substrate. This property makes it very useful for measuring concentration based on how much light it produces,

subsequently, making it an excellent reporter gene. To isolate the uORFs, PCR was performed on cDNA from *N. crassa* using primers designed to amplify each uORF with its native start codon context and to introduce cloning sites at the 5' and 3' end of each amplified fragment. Each candidate uORF was placed in the 5' UTR of a luciferase gene in pJW201 vector plasmid designed to produce synthetic mRNA for *in vitro* translation experiments. mRNA containing these uORFs were tested for their ability to produce luciferase in comparison to mRNA lacking uORFs.

The mechanism by which important genes, such as eIF5 and I3PS, are regulated can shed new light on gene control and balance. The more known about genes regulation the better gene expression can be manipulated and the more precise medical treatments become to target diseases caused by expression imbalances. As new information on gene control emerges the better our understanding of the processes in common for all eukaryotic organisms.

CHAPTER II

METHODS

The sequences of the uORFs in question can be found on-line at www.ncbi.nlm.nih.gov at the 5‘ end of I3PS, numbered NCU06666, and eIF5, numbered NCU00366. pJW201, used to make the constructs, was designed for *in vitro* experiments and was provided to by Jiajie Wei in the lab (see the plasmid map in the figure on page 9). Qiagen Kits 28104 and 20051 were used for PCR purification and gel purification respectively. All enzymes and buffers used for cloning were obtained from New England BioLabs. Passive lysis buffer was provided by Promega. All other materials were of analytical grade or higher and obtained from Sigma, Fisher or Invitrogen.

PCR

Six PCR primers (two for each uORF) were designed to amplify the uORF and introduce new BglII and XhoI restriction sites to insert the products into pJW201. PCR was performed with the following reagents and amounts: 14.8µL water, 2µL 5ng/µL cDNA, 1.25µL 10µM Primer 1, 1.25µL 10µM Primer 2, 2.5µL 10x PCR Buffer, 2µL 2.5mM dNTPs, 0.2µL 5U/µL Taq and 1µL 5mM Mg^{2+} for a final concentration of 1.7mM Mg^{2+} in a final volume of 25µL. Once the program finished (for program specifications see Appendix A), the fragments were run on a 1% agarose gel to make sure the desired products were made. The PCR products were purified by Qiagen Kit 28104 and

concentrations were determined by Nano Drop and were approximately 600ng/µL in 20µL volume.

Restriction enzyme digestion

The digestion consisted of 8.5µL PCR DNA, 2.5µL 10x Digestion Buffer IV, 1.0µL 10kU/mL BglII, 0.5µL 20kU/mL XhoI, 0.25µL 10mg/mL BSA and 12.25µL water. The reaction was incubated for two hours at 37ºC and the appropriately sized products were gel-purified using a 1% Agarose gel and a Qiagen kit 20051. The same was done for pJW201 vector plasmid with the following reagent amounts: 10µL 1µg/µL vector DNA, 15µL 10x Digestion Buffer IV, 10µL 10kU/mL BglII, 5.0µL 20kU/mL XhoI, 1.5µL 10mg/mL BSA and 109.5µL water. The vector was also gel-purified and water was added to each purified DNA fragment to produce final concentrations of 340ng/µL PCR insert and 670ng/µL pJW201.

Ligation

The PCR insert was ligated into pJW201 using the reaction: 5.0µL water, 1.0µL 10x T4 ligation buffer, 1.0µL 340ng/µL insert, 2.5µL 670ng/µL pJW201 and 0.5µL 400kU/mL T4 Ligase for a total volume of 10µL. The reaction was run at room temperature overnight then stored at -20ºC until used for transformation. The resulting plasmids were numbered p2 (I3PS uORF), p4 (eIF5 uORF 1) and p6 (eIF5 uORF 2).

Transformation

The plasmids were used to transform 25µL of DH5a chemically competent *Escherichia coli* strains. Once the competent cells were thawed, 5µL of the ligation mixture was added and incubated on ice for 30 minutes. The bacteria were heat shocked at 42°C for one minute and put back on ice for two minutes. 500µL LB media was added to the cells and incubated at 37°C for 1 hour. After this time the bacteria were plated on LB ampicillin media and left to grow overnight at 37°C. Single colonies were selected and grown in 2mL ampicillin LB overnight at 37°C. The plasmids were purified from these new cultures, assessed for identity by EcoRI digestion and sequenced at a commercial facility to ensure the fidelity of the uORF sequence (see Appendix A for the mini prep protocol). After sequencing, the correct clones were grown in 100mL ampicillin LB and the plasmids were purified by a standard midi prep protocol (see Appendix A) followed by two rounds of 50:50 phenol:chloroform extraction. DNA was ethanol precipitated and dissolved in water. The concentration of the plasmids were determined by Nano Drop and adjusted to 2µg/µL.

Transcription

Each plasmid was linearized with EcoRI using the following reagents: 5.0µL 2µg/µL DNA, 10µL 10x Digestion Buffer I, 5.0µL EcoRI and 85µL water. The DNA was ethanol precipitated and resuspended in 10µL water to a final concentration of 1µg/µL. 3.0µL of this 1µg/µL linearized DNA was then added to 5.0µL 5x Transcription Buffer,

2.5µL NTPs (30mM ATP, CTP, UTP and 6mM GTP) and 0.5µL 1M DTT, and pre-incubated at 37°C for 2 minutes. After pre-incubation 14µL of reaction mix 2 (5.5µL DEPC water, 0.5µL 0.1M spermidine, 6.0µL 10mM Cap, 0.5µL 40U/µL RNasin Plus, 1.5µL 20U/µL T7 RNA Pol) was added and incubated for 2 hours at 37ºC. The product mRNA was precipitated by adding 110µL DEPC water, 15µL 5M ammonia acetate and 150µL isopropanol to the original reaction mix and incubating it at -80ºC for 20+ minutes. The precipitate was collected after centrifugation at 4ºC for 20 minutes at 14000 rpm, ethanol washed, resuspended in 10µL DEPC water and a small sample run on a gel for fidelity and concentration measurements. The mRNA was adjusted to 60ng/µL and stored at -80ºC.

***In vitro* translation**

Translation reactions were done in triplicate on p2, p4 and p6 mRNA from three different batches made from the same template DNA, and a T7 Luciferase control to make a total of 30 reactions. There were three ingredients needed for this reaction: 4µL mixture 1 (2.27µL DEPC-treated water, 1µL 10x energy mix (10mM ATP, 2.5mM GTP, 250mM creatine phosphate), 0.06µL 10 U/µL creatine phosphokinase, 0.35µL 2M potassium acetate, 0.12µL 0.1M MgAc, 0.1µL 1mM amino acid mix, 0.1µL 40U/µL RNasin Plus), 1µL 6ng/µL mRNA and 5µL *N. crassa* extract. The mRNA was added to Mixture 1 with the extract added last, and the reaction mixtures were then incubated for

30 minute at 26ºC. 50µL of 1.2x passive lysis buffer was then added after 30 minutes to stop each reaction.

Luciferase assay

A Perkin Elmer Victor3V Wallace 1420 Multilabel Counter machine was use to perform the Luciferase assay. 15µL of each sample was loaded into an individual well on a well plate and placed in the machine. 50µL of firefly luciferace assay reagent was automatically added at the appropriate times to each well. Luminescence was measured in Relative Light Units (RLU).

CHAPTER III

RESULTS AND DISCUSSION

PCR results

PCR was performed on cDNA to amplify the uORFs of eIF5 and I3PS with BglII sites created at their 5' ends and XhoI sites at their 3' ends to orient the uORFs in the correct direction and frame with respect to the gene. These two sites were chosen as the cloning sites since they were oriented in such a way as to replace the Arginine Attenuator Peptide (AAP) uORF already in pJW201 with the uORF inserts of eIF5 and I3PS. Figure 2 shows the restriction map of pJW201 with the restriction sites, used to clone the inserts, circled in red. By using these sites the AAP can be removed and the new uORFs inserted cleanly.

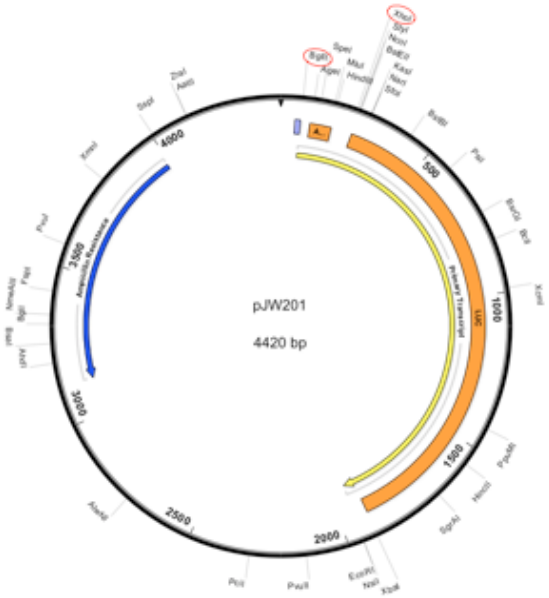

Figure 2. Restriction map of pJW201.
The restriction sites used to insert the uORFs were BglII and XhoI, circled in red.

The magnesium content of the PCR reaction had to be optimized to obtain a maximum yield. The 10x PCR reaction buffer contains 15mM Mg^{2+} resulting in a reaction with a fixed concentration of 1.5mM magnesium. When run at this concentration of magnesium there was no product, therefore the concentration was experimentally adjusted to determine the optimal magnesium concentration. Two tests were done on two PCR reactions, one with 1.7mM and the other with 1.9mM magnesium concentrations, obtained by adding additional magnesium into the reaction. The results are shown in Figure 3 below. The left gel is of the reaction with 1.9mM magnesium while the right gel is the 1.7mM magnesium run. The 1.7mM magnesium PCR reaction performed the best, therefore those products were used in the digestion reaction.

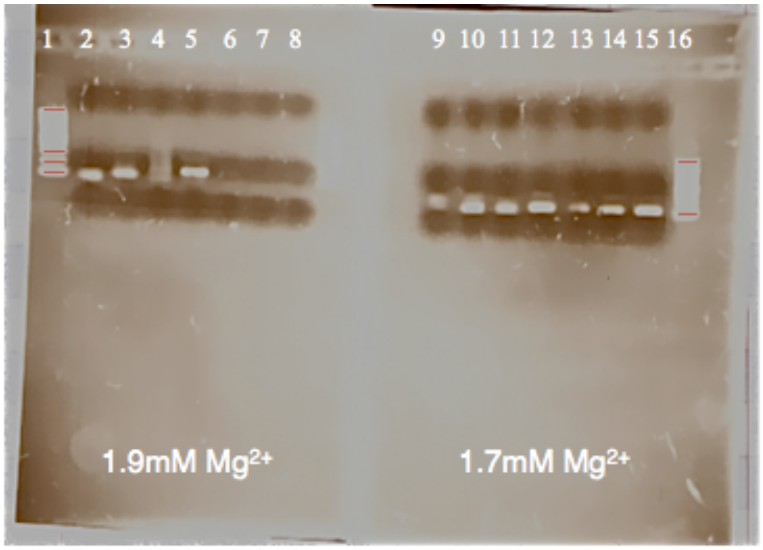

Figure 3. Results from PCR.
The left gel is the PCR product from the reaction with 1.9mM magnesium. The right gel is the PCR product from the reaction with 1.7mM magnesium. The red lines mark bands on the molecular weight marker. Lane 1 contains the molecular weight marker, lane 2 is cDNA control, lane 3 is *in vitro* eIF5 second uORF, lane 4 is *in vivo* eIF5 second uORF, lane 5 is *in vitro* eIF5 first uORF, lane 6 is *in vivo* eIF5 first uORF, lane 7 is *in vitro* I3PS uORF and lane 8 is i*n vivo* I3PS uORF. Lanes 9-15 are in the same order as 2-8 with lane 16 being the molecular weight marker. Both *in vitro* and *in vivo* designated uORF's are shown on this gel.

Enzyme digestion

Enzyme digestion was performed to linearize and remove the AAP insert in the pJW201 vector, and to produce the 5' "sticky ends" in both the PCR products and the vector needed for ligation. Digestion was confirmed successful during gel purification when the bands corresponding to the expected fragment sizes (approximately 4.4kb for JW201 and 100bp for the PCR inserts) were observed and removed from the gel. This ensures that the fragments used for ligation are free of any contaminating DNA molecules that may reduce the ligation efficiency, and helps ensure that the appropriate fragments are used in the ligation.

Plasmid identification

After transformation the purified plasmid's identity needed to be assessed. The plasmids were cut with EcoRI and run on a 1% agarose gel. A single band was expected at approximately 4.5kb. The gel photographs are shown in Figure 4 below.

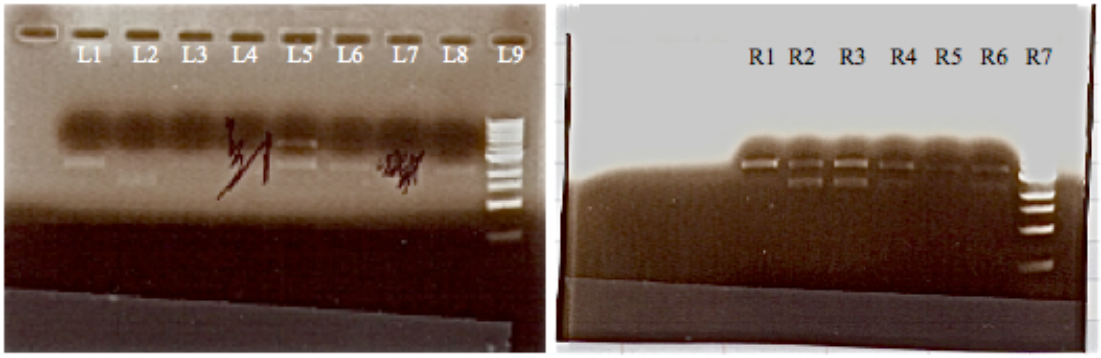

Figure 4. Plasmid identification gel.
The left gel was run with p4 and p6 while the right gel was run with p2. Lane L1 is p6-4, lane L2 is p6-3, lane L3 is p6-2, lane L4 is p6-1, lane L5 is p4-4, lane L6 is p4-3, lane L7 is p4-2, lane L8 is p4-1, lane L9 is the molecular weight marker, lane R1 is p2-6, lane R2 is p2-5, lane R3 is p2-4, lane R4 is p2-3, lane R5 is p2-2, lane R6 is p2-1, and lane R7 is molecular weight marker.

Lanes L4 and L7 did not have any observable product, thus the marking over the lane. Only lanes with one band at 4.5kb and an observable signal were chosen to be sequenced. Thus, the plasmids corresponding to lanes L2, L4, L7, L9, and R7 were sent for sequencing. The sequences from the uORFs in the plasmids corresponding to lane T4, T9 and S7 were as predicted, so these plasmids were used for transcription.

Transcription

The synthetic mRNA was run on a gel and scanned using a Typhoon 9400 to quantify and assess the fidelity of the mRNA. A total of three mRNA batches were made over the course of this project. The undiluted mRNA of batch one and two (Lane 5-10) and the 60ng/µL stock of the third batch (lane 2-4) were used (refer to figure 5).

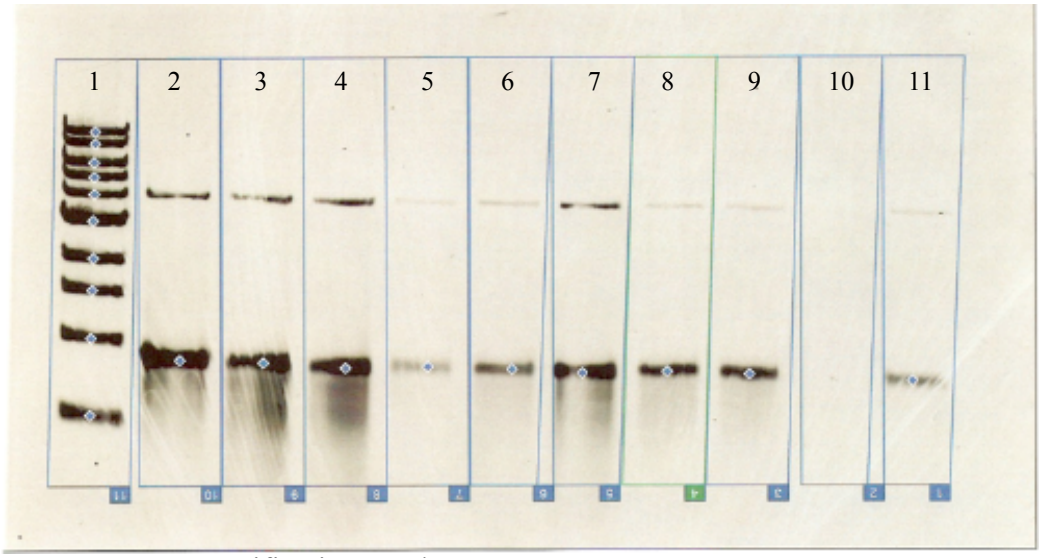

Figure 5. mRNA quantification results.
Lane 1 contains the molecular weight marker, lane 2 is batch 1 p2 mRNA, lane 3 is batch 1 p4 mRNA, lane 4 is batch 1 p6 mRNA, lane 5 is batch 2 p2 mRNA, lane 6 is batch 2 p4 mRNA, lane 7 is batch 2 p6 mRNA, lane 8 is batch 3 p2 mRNA, lane 9 is batch 3 p4 mRNA, lane 10 is batch 3 p6 mRNA and lane 11 is the T7 control mRNA.

All of the batches had some degradation, with the older batch 1 and 2 having the most. Lane 2 is blank due to loading error, so its concentration was estimated based on the concentrations in lane 3 and 4 since their concentrations were previously determined and diluted to 60ng/µL and there was insignificant differences between the two lanes. These data show that all of the mRNA is useable and of the right size. The stock mRNAs were normalized to a fixed concentration of mRNA by adding water as appropriate, producing concentrations of 6ng/µL.

Luciferase assay

The results from the luciferase assay are summarized in Figure 6 below. The T7 control is made from pJW201 without an uORF in the 5'UTR of the luciferase mRNA, allowing it to be translated without any uORF-mediated regulation, and appeared highest as would be expected. I3PS uORF had lower regulation (higher luminescence) in older batches 1 and 2 of mRNA than in the new batch 3 (refer to figure on page 21 in Appendix A). This varying expression of I3PS's uORF due to age can not readily be explained, but all of the batches showed some regulation though lower than eIF5 uORFs. This can be accounted for by the fact that the I3PS uORF does not have an AUG start codon where as both eIF5 uORFs do so the uORF does not get recognized by the scanning ribosome as efficiently. Both of the eIF5 uORF's show strong regulation, consistent between all the batches. Batch 1 and 2 eIF5 mRNAs show slightly varying expression but nothing statistically

significant at the 95% confidence level. These results are not unexpected since the eIF5 uORF's have AUG start codons predisposing them for higher ribosome initiation.

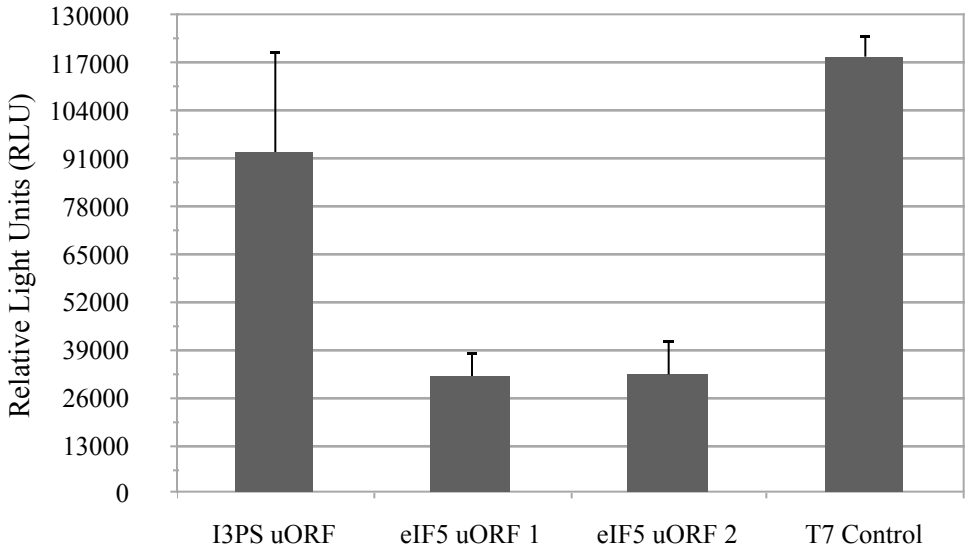

Figure 6. Average expression of Luciferase measured by Luminescence.
The Y-axis shows the luminescence of the Luciferase system measured in relative light units (RLU). Each of three batches of uORF inserts were tested in triplicate then averaged together to give the graph. Error bars were inserted based on the standard deviation of the data.

Toeprinting data

Cheng Wu performed toeprinting analysis on the uORFs to map the position of the ribosome at rate-limiting steps in translation. Data from his experiments (unpublished) are shown in Figure 7 below. The bottom asterisk indicates the ORF start codon initiating translation. Any asterisk above that correlates to translation initiation at an upstream start sequence. I3PS uORF shows weak initiation at its alternative start codon at time 0 and 10 minutes. This is expected since its initiation codon is not the normal AUG codon. Both eIF5 uORF 1 and eIF5 uORF 2 shows strong initiation at their

uAUG. The data for eIF5 uORF 2 indicates that initiation can occur at either of its in frame uAUG codons. This data confirms that these uORFs function at translation. Though which uAUG in eIF5 uORF 2 functions as the start site is not the focus of this paper, these data suggest that this uORF should have more regulatory activity than eIF5 uORF 1. This phenomena will have to be explored at a later time.

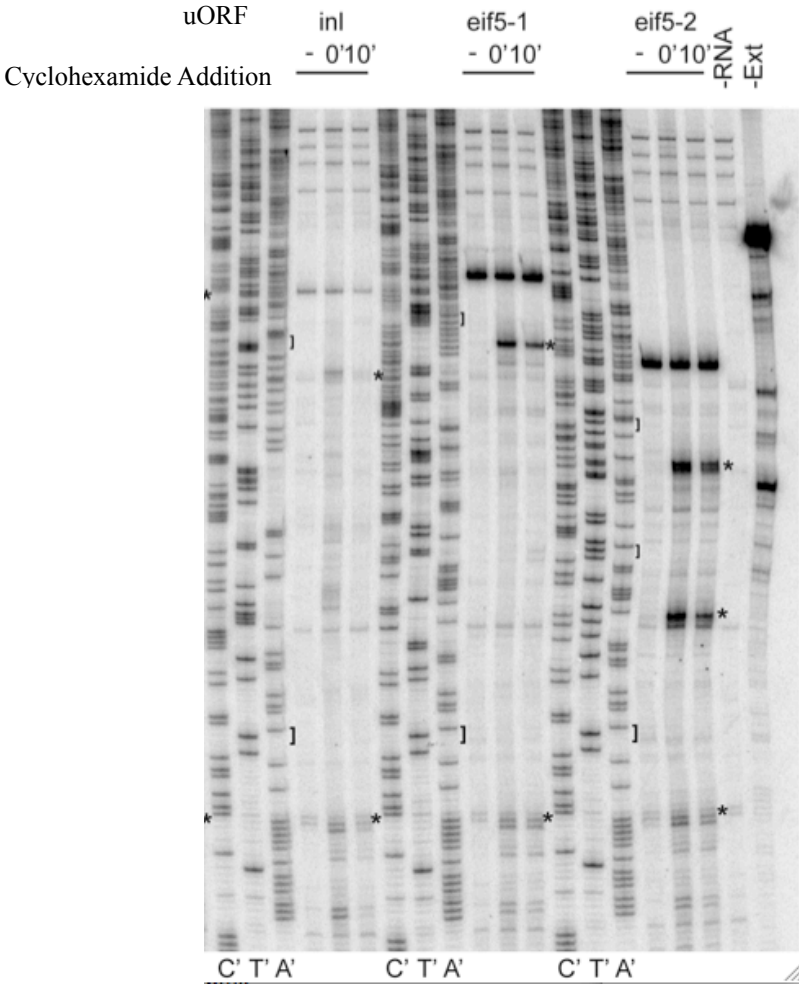

Figure 7. Toeprinting data.

The bottom asterisks correlate to initiation at the ORF start codon while the asterisks above those are ribosome initiation at upstream sites. The columns labeled C', T', and A' indicate nucleotide sequence with a G assumed anywhere a band is not found for A, T or C. The time points are the points when cycloheximide was added to stop the translation reaction and lock the ribosome in its position, indicated by the bands with asterisks next to them. The - time point indicates no extract was added and serves as a control for how the lanes should look without any ribosome initiation.

CHAPTER IV

CONCLUSIONS

The data shown in Figure 6 and Figure 7 suggests that I3PS uORF and both eIF5 uORFs have regulatory activity, therefore both of the uORF's in eIF5 contribute to the effective reduction in gene expression. Both of the uORFs in eIF5 can reduce expression of Luciferase by 65%. Thus, these are potent regulatory uORFs. If these acted independently, their combine regulation would severely knock down the eIF5 levels. Either of eIF5 uORF 2 uAUGs can function as an initiation site, implying that it could be a stronger regulator than eIF5 uORF 1. This will be explored subsequently.

From these data it can be concluded that these uORFs can act as translational regulators of their respective genes. I3PS regulatory activity is suggestive while both of eIF5's uORF are powerful regulators. However, these sequences are demonstrated to be regulatory only in *in vitro* systems outside of the cell. Work is being done on *in vivo* expression to help support the *in vitro* data or provide a new point of study for how the cell can suppress the uORF's activity.

A next step is to look at how important the start codon context of the uORFs is in its regulatory activity. Data from this test would shed light on how these uORFs function as regulatory elements.

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APPENDIX A

Protocols

PCR program

The reaction was run in an Eppendorf Mastercycler Gradient PCR machine. One round at 94ºC for two minutes. 35 rounds of: 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 45 seconds. One round at 72ºC for five minutes. Stationary at 4ºC until retrieved.

Mini prep

Transfer 1.5mL of the culture to a centrifuge tube and pellet cells for 15 seconds at 13.2krpm. Resuspend the pellet in 150µL cold Solution I and 5µL RNase. Add 350µL Solution II and invert the mixture until it clears then incubate at room temperature for five minutes. Add 250µL cold Solution III, handshake well and incubate on ice for 10 minutes. Centrifuge for 10 minutes at 4ºC at 13.2krpm. Transfer the supernatant to a new tube and add isopropanol at a 1:1 volume ratio, mix and centrifuge at 13.2krpm for five minutes. Wash the pellet with 500µL 70% ethanol then dry the pellet for five minutes. Resuspend the pellet in 15µL water and store at -20ºC.

Solution I: 50mM Glucose 25mM Tris pH 8.0 10mM EDTA

Solution II: 0.2M NaOH 1% SDS

Solution III: 3M Potassium Acetate 11.5% Glacial Acetic Acid

Midi prep

Pellet cells at 8krpm in a GSA rotor for 10 minutes at 4ºC then suspend the cells in Solution I. Add four mL of Solution II and invert to mix until solution clears. Incubate at room temperature for five minutes then add three mL ice-cold Solution III and incubate on ice for 10 minutes. Centrifuge for 15 minutes at 4ºC at 5krpm then transfer the supernatant to a 15mL tube and add 0.6 volume isopropanol and invert to mix. Centrifuge at 10krpm in an SS-34 rotor for 10 minutes at 4ºC. Transfer the supernatant to a new 15mL tube and add equal volume isopropanol. Centrifuge again at 10krpm for 10 minutes in an SS-34 rotor at room temperature. Wash the pellet with 70% ethanol then resuspend it in 400µL TE pH 8.0 containing 20µg/mL RNase A and transfer it to a microcentrifuge tube. Incubate it for 30 minutes at room temperature. Extract once with phenol:chloroform and once with chloroform. Repeat phenol:chloroform extraction then ethanol precipitate the DNA. Dissolve the DNA in water or TE pH 8.0.

Additional data

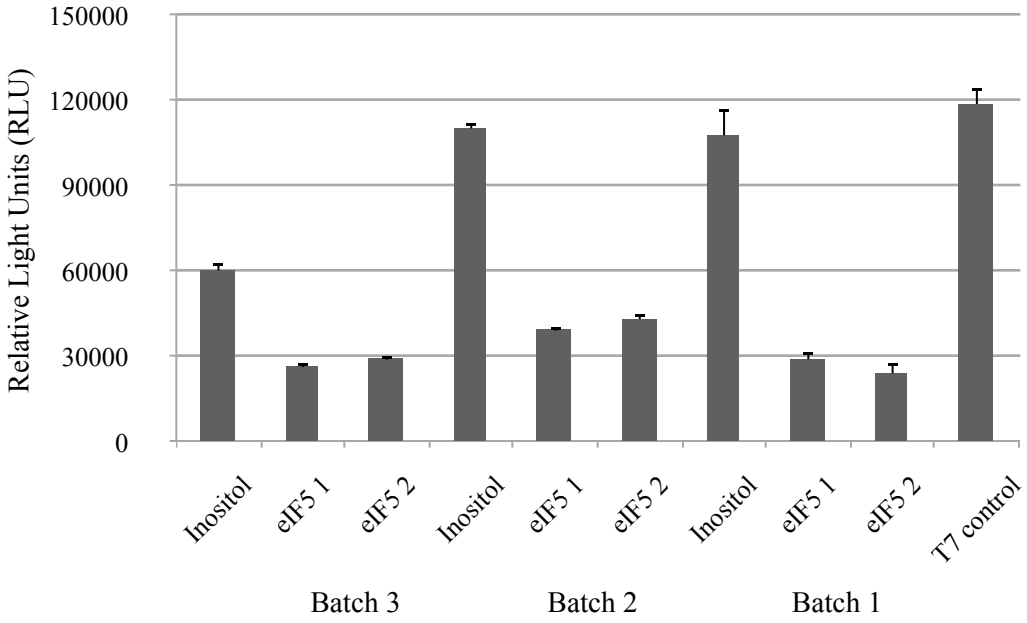

Figure 8. Expression of Luciferase measured b Luminescence in each batch.
The Y-axis shows the luminescence of the Luciferase system. Each uORF insert was tested in triplicate then averaged together to give the graph. Error bars were inserted based on the standard deviation of the data. Batch 1 is the newest batch with 2 and 3 being three months old.

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