TRANSLATIONAL REGULATION OF GROWTH ARREST AND DNA DAMAGE-INDUCIBLE GENE GADD34 VIA ITS 5’ UNTRANSLATED REGION UPSTREAM OPEN READING FRAME DURING EUKARYOTIC INITIATION FACTOR 2 ALPHA PHOSPHORYLATION

by

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ABSTRACT

Endoplasmic reticulum (ER) stress activates an integrated stress response which causes inhibition of overall protein synthesis via phosphorylation of the eukaryotic initiation factor 2alpha (eIF2alpha). However, ER stress also results in selective translation of mRNAs, one of which is a transcription factor ATF4. ATF4 activates transcription of downstream stress-induced genes such as growth arrest and DNA-damage inducible gene 34 (GADD34) under ER stress. The function of GADD34 is to dephosphorylate eIF2alpha by interacting with protein phosphatase 1, thus leading to recovery of overall protein synthesis and translation of stress-induced transcripts through a negative feedback mechanism. In this thesis, we showed that GADD34 is not only transcriptionally induced, but also translationally regulated for maximal expression under ER stress. Translational regulation of GADD34 was mediated by its 5’ untranslated region (5’ UTR), which was found to contain two upstream open reading frames (uORFs) in human and mouse. It was revealed that the downstream uORF2 is required for basal repression and translational upregulation under ER stress, while the upstream uORF1 is dispensable in this regulation. In addition, the uORF2 is readily recognized and translated, but the uORF1 is bypassed by the scanning ribosomes. Further mutational analysis on the GADD34 5’ UTR demonstrated that the uORF2 and the intercistronic region between the uORF2 and the main ORF are sufficient to direct translation when eIF2alpha is phosphorylated. In this process, the amino acid/nucleotide identity of the uORF2 was not required, but its conserved size was important. The sequence conservation within the intercistronic region also was identified, but changing the length
and pyrimidine:purine ratio in this region did not significantly affect translational regulation. Finally, we set up in vitro translation systems where cap-dependent translation is compromised by inhibiting ternary complex and eIF4F formation in order to test GADD34 translational regulation. The results from the current thesis suggest that GADD34 translation is mediated through its 5’ UTR via a unique mechanism, which may serve as a model to understand translational regulation of other uORFs-containing mRNAs under cellular stress.
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LIST OF ABBREVIATIONS

2AP  2-Aminopurine
4E-BP  eIF4E binding protein
5’ UTR  5 prime untranslated region
ATF  Activating transcription factor
CrPV  Cricket paralysis virus
DMEM  Dulbecco’s modified Eagle medium
DNA  Deoxyribonucleic acid
dsRNA  Double stranded RNA
DTT  Dithiothreitol
ECL  Enhanced chemiluminescence
EDTA  Ethylenediaminetetraacetic acid
eIFs  Eukaryotic initiation factors
ER  Endoplasmic reticulum
GADD34  Growth arrest and DNA damage-inducible gene 34
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>GCN</td>
<td>General control non-depressible</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRI</td>
<td>Heme-regulated inhibitor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IGR</td>
<td>Intergenic region</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol requirement 1</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>Met-tRNAi</td>
<td>Initiator methionine transfer RNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase R-like ER kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>PKR</td>
<td>Protein Kinase R</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RRL</td>
<td>Rabbit reticulocyte lysate</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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</table>
I would like to thank my supervisor, Dr. Eric Jan for giving me the opportunity to work under him on this intriguing project and for his continuous guidance and support throughout the course of my graduate career. His extraordinary passion for science has truly been inspirational, and taught me to be more appreciative of scientific research. I would also like to thank Dr. Jim Johnson, Dr. Ivan Sadowski, and Dr. George Mackie for being in my thesis committee and for providing valuable advice.

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DEDICATIONS

To my loving parents, Hyun Taek Lee and Myung Jin Kang
CHAPTER I

Introduction

1.1 Translational Regulation during Cellular Stresses

When cells encounter environmental stresses, one of the first responses is translational shutoff (Ron and Harding, 2007). In most cases, the initiation step of translation is targeted through the modification of initiation factors and/or alteration of their cellular abundance (Mohr et al., 2007; Ron and Harding, 2007). It appears that the activities of the translational factors eIF2 and eIF4F are often targeted to inhibit overall protein synthesis (Clemens, 2001). Because translation is an energetically demanding process, inhibition of overall translation can greatly conserve cellular energy until the cellular stress subsides and the cell recovers (Holcik and Sonenberg, 2005; Pannevis and Houlihan, 1992). The shutoff of translation is also a means to reprogram gene expression through the selective translation of a subset of mRNAs which utilize noncanonical mechanisms to bypass the translational block. These mRNAs often encode proteins important for the cell to survive or adapt (Holcik and Sonenberg, 2005). My thesis focuses on how select mRNAs bypass translational inhibition and are translated during cellular stress. Here, we address this question by studying translational regulation of *GADD34*, one of the regulatory genes involved in recovery of overall protein synthesis following ER stress.
1.2 Initiation of Translation

For the majority of mRNAs in eukaryotic cells, translation initiation involves recruitment of the 40S ribosomal subunit to the 5' cap structure of the mRNA (Figure 1.1) (Pestova et al., 2007). This process is mediated by a number of initiation factors. Specifically, the eIF4F complex, which consists of the cap binding protein eIF4E, the scaffold protein eIF4G and the RNA helicase eIF4A, is recruited along with eIF4B, a cofactor for eIF4A, to the 5' cap (m$^7$GTP) of the mRNA. eIF3 recruits the 40S subunit and the ternary complex (TC) (eIF2·GTP·Met-tRNA$^\text{Met}_\text{i}$) via an interaction with eIF4G. The poly (A) binding protein (PABP), which binds to the 3' poly A tail, also interacts with eIF4G to circularize the mRNA and promotes eIF4F binding to the 5' cap. The 40S complex then scans the mRNA for an AUG start codon, where eIF5 induces hydrolysis of GTP bound to eIF2, resulting in the release of eIF2·GDP and other eIFs (Pestova et al., 2007). Finally, 60S subunit joining is catalyzed by eIF5B·GTP and GTP hydrolysis to form an elongation-competent 80S ribosome (Pestova et al., 2000).

1.3 Phosphorylation of eIF2alpha

After recognition of the AUG codon, GTP bound to eIF2 is hydrolyzed, resulting in the release of the Met-tRNA$^\text{Met}_\text{i}$ and dissociation of eIF2 from the 40S. In order for eIF2 to take part in another round of translation, eIF2 must be recycled to the GTP form, which can then bind to another ternary complex for the next round of translation. This GDP to GTP exchange is mediated by a guanine nucleotide exchange factor, eIF2B
Figure 1.1  Cap-Dependent Translation Initiation

The diagram describes the nine steps leading to formation of 80S translation initiation complex during cap-dependent translation initiation. (1) A ternary complex is formed with eIF2, GTP, and initiator Met-tRNA, and (2) forms a 43S preinitiation complex with 40S subunit, eIF3, eIF1, and eIF1A. (3) eIF4F is recruited to the 5’cap of mRNA, and the mRNA is “circularized” via interaction between PABP and eIF4G. (4) The 43S preinitiation complex is then recruited the cap, and (5) this complex scans the transcript for an initiation codon. (6) Once the start codon is recognized in the P site of the 40S, and basepaired with the initiator Met-tRNA, GTP on eIF2 is hydrolyzed to GDP by eIF5, and eIF2-GDP and other initiation factors are released from the transcript. (7) 60S ribosomal subunit binding is facilitated by eIF5B-mediated GTP hydrolysis, and (8) following the release of eIF5B, 80S initiation complex formation is complete. (9) At the same time, eIF2-GDP is recycled back to the GTP-bound form by eIF2B (Pestova et al., 2007 modified by permission).
(Figure 1.2) (Nika et al., 2000; Pestova et al., 2007; Williams et al., 2001). The activity of eIF2, which consists of three subunits, alpha, beta, and gamma, can be inhibited by phosphorylation of serine 51 of the alpha subunit. eIF2B binds to the phosphorylated eIF2alpha with much greater affinity, approximately 150 fold higher than to non-phosphorylated eIF2alpha (Rowlands et al., 1988). Thus, phosphorylated eIF2alpha acts as a competitive inhibitor of eIF2B and inhibits the recycling of eIF2 (Dever et al., 1995; Hinnebusch et al., 2007; Rowlands et al., 1988). Furthermore, the ratio of eIF2 to eIF2B is generally high (i.e. 7:1 in reticulocyte lysates) in cells, making eIF2B a limiting factor in inhibition of translation via eIF2alpha phosphorylation (Oldfield et al., 1994; Pavitt, 2005; Webb and Proud, 1997). As a result, relatively small increases in eIF2alpha phosphorylation can lead to an inhibition of both eIF2B activity and overall protein synthesis.

1.4 eIF2alpha Kinases

eIF2alpha can be phosphorylated by four known mammalian eIF2alpha kinases: PKR, PERK, HRI, and GCN2 (Dever et al., 2007). They all share a conserved eIF2alpha kinase domain with approximately 25-37% amino acid sequence identity. The kinase activity is regulated through a unique regulatory domain, which senses distinct cellular stresses. Upon activation, each dimerizes and undergoes autophosphorylation leading to phosphorylation of eIF2alpha (Dever et al., 2007). I will briefly describe the regulation and activation of these four eIF2alpha kinases.
eIF2 is a trimer consisting of alpha, beta, and gamma subunits, and forms a ternary complex (TC) with GTP and Met-tRNA$_{\text{Met}}$. Upon recognition of an initiation codon during translation initiation, GTP on TC is hydrolyzed by eIF5. GDP-eIF2 is recycled back to the GTP-bound form by eIF2B, a guanine nucleotide exchange factor, for the next round of initiation. Under viral infection or stress, eIF2alpha is phosphorylated by eIF2alpha kinases. The phosphorylated eIF2 now acts as a competitive inhibitor of eIF2B, thereby preventing formation of TC for translation initiation. Figure courtesy of Dr. E. Jan.

**Figure 1.2  Inhibition of Translation Initiation by eIF2alpha Phosphorylation**
PERK, found in both vertebrates and invertebrates, localizes on the ER membrane, and is important in regulating the ER homeostasis by sensing the amount of unfolded proteins in the ER (Dever et al., 2007). When there is an accumulation of misfolded proteins in the ER lumen, PERK is activated through oligomerization and autophosphorylation, leading to phosphorylation of eIF2alpha (Figure 1.3) (Harding et al., 1999). Its activation is thought to be regulated through an interaction of its amino-terminal region with ER chaperones such as BiP (GRP78) and GRP94 (Bertolotti et al., 2000; Ma et al., 2002). Upon accumulation of unfolded proteins, ER chaperones are titrated away, thus allowing PERK to dimerize and be activated (Bertolotti et al., 2000). In addition, the amino-terminal luminal regulatory region of PERK, which is homologous to the corresponding region in IRE1, has been proposed to bind to unfolded proteins (Credle et al., 2005). The deletion of this region disrupts oligomerization and activation of PERK under ER stress, indicating that this region is important for activation (Ma et al., 2002). Paradoxically, activated PERK and eIF2alpha phosphorylation lead to the translation of select mRNAs, some of which encode transcription factors that activate downstream stress-response genes (Scheuner and Kaufman, 2008).

The eIF2alpha kinase, PKR, is a central component in the host antiviral response. PKR contains two dsRNA-binding motifs (dsRBMs), which bind to dsRNA 30 to 85 bp in length (Dever et al., 2007; Nicholson, 1996). During viral infection, binding of viral dsRNA to the dsRBMs induces a conformation change and promotes dimerization of PKR, thereby activating the kinase (Figure 1.3) (Dever et al., 2007; Green and Mathews, 1992; Wu and Kaufman, 1997; Zhang et al., 2001). This is thought to inhibit overall translation by eIF2alpha phosphorylation to prevent viral gene expression and to promote
Figure 1.3   Four eIF2alpha Kinases
PKR is dimerized and activated via autophosphorylation by binding to dsRNA. PERK is basally kept inactive via interaction with BiP, an ER chaperone. Unfolded proteins titrate BiP away from PERK and/or bind to IRE1 homology region of PERK to dimerize and activate PERK. Interactions among CTD, HisRS and kinase domains keep GCN2 basally inactive, while under amino acid starvation, uncharged tRNA binds to the CTD+HisRS domains, and this induces autophosphorylation and activation of GCN2. In the presence of heme, interaction between the amino terminal heme binding site (grey) and the kinase domain (light blue) represses the kinase activity, but under heme deprivation, this interaction is disrupted, and HRI is activated (Dever et al., 2007).
apoptosis of the infected cells (Ron and Harding, 2007). PKR expression is also induced by interferon, which is secreted by virally infected cells (Beretta et al., 1996; Kostura and Mathews, 1989). The upregulation of PKR sensitizes cells to virus infection and mediates an antiviral state of the cell. However, viruses are found to produce inhibitors against PKR to resist its antiviral activity (Mohr et al., 2007). The fact that viruses have evolved mechanisms to counteract PKR attests to the importance of PKR in the host immune response.

HRI, heme-regulated inhibitor, is found in vertebrates and in *Schizosaccharomyces pombe*, and is the main kinase in erythroid cells (Lu et al., 2001; Zhan et al., 2002). Normally, the amount of globin proteins has to be balanced to the amount of heme in erythroid cells to produce the optimal amount of haemoglobin (Ron and Harding, 2007). If heme is lacking, globin production has to be inhibited. Since erythroid cells are enucleated, the sole control of globin production is at the level of translation, and HRI plays an important role in this response. In the absence of heme, HRI is activated, thus leading to eIF2alpha phosphorylation and shutoff of globin synthesis. Normally, the amino-terminal region of HRI binds to heme, which can interact with the kinase domain to inhibit its activity (Dever et al., 2007; Yun et al., 2005). Heme deprivation disrupts this interaction, and induces a conformational change within the amino terminal domain, thereby turning on the kinase activity of HRI (Figure 1.3). HRI has also been shown to be activated under a number of stresses including arsenite exposure, heat shock and osmotic stress, yet how these stresses activate HRI remains to be investigated (Lu et al., 2001; McEwen et al., 2005).
Another eIF2alpha kinase, GCN2, senses amino acid deficiency, and is found in all eukaryotes (Dever et al., 2007). GCN2 kinase activity is known to be regulated through interdomain interactions between its carboxy-terminal domain (CTD), a domain resembling histidyl-tRNA synthetase (HisRS), and a protein kinase domain. The interactions between these domains inhibit GCN2 kinase activity under basal condition (Qiu et al., 2001). When amino acid levels are low in the cell, uncharged tRNAs accumulate and can bind to the CTD+HisRS domain, thus disrupting its interaction with the kinase domain (Dong et al., 2000). GCN2 can now dimerize, undergo autophosphorylation, and phosphorylate eIF2alpha (Figure 1.3) (Dever et al., 2007). GCN2-mediated eIF2alpha phosphorylation inhibits overall protein synthesis until the cellular amino acid levels recover. However, translational repression via eIF2alpha phosphorylation also increases the translation of select mRNAs. The most studied example is yeast GCN4, which is a transcriptional activator of genes for amino acid biosynthesis (Hinnebusch, 2005). Thus, the cell has evolved mechanisms to actively synthesize enzymes for amino acid biosynthesis and thereby promote its recovery.

1.5 ER Stress Pathway

The proper functioning of the ER is crucial for cellular viability, as close to one third of total cellular proteins is synthesized and processed in the ER (Ghaemmaghami et al., 2003). Newly-synthesized secretory and membrane-bound proteins are folded by ER-resident chaperones and folding catalysts in the ER before they are trafficked to the Golgi apparatus (Scheuner and Kaufman, 2008). Under conditions when unfolded proteins
accumulate, the cell responds by activating a series of downstream signalling cascades collectively called the unfolded protein response (UPR) (Scheuner and Kaufman, 2008). The UPR consists of three main arms mediated by three ER-resident proteins, PERK, IRE1α, and ATF6. All three arms mediate distinct signalling events, and eventually lead to the transcription of downstream stress response genes encoding chaperones and ER-associated degradation (ERAD) proteins (Scheuner and Kaufman, 2008). The UPR is an adaptive cellular response which is crucial in regulation of cell survival and recovery during ER stress (Hu et al., 2007).

The accumulation of unfolded proteins in the ER can be induced by several ways. These include increased overall translation exceeding a normal capacity of the ER, defective processing of newly synthesized proteins, and mutations that cause misfolding of proteins (Ermonval et al., 1997; Scheuner et al., 2005; Umebayashi et al., 1997). More recent studies have also suggested that accumulation of lipids and cholesterol and amino acid deprivation result in ER stress and activation of the UPR (Abcouwer et al., 2002; Pineau et al., 2009). Several chemical agents can artificially cause ER stress in cells. Thapsigargin is one of the most commonly used ER stress inducers, which is a sesquiterpene lactone extracted from a plant, *Thapsia garganica* (Christensen et al., 1982). Thapsigargin is a selective inhibitor of the ER Ca\(^{2+}\)-ATPase that blocks Ca\(^{2+}\) transport, thus depleting Ca\(^{2+}\) in the ER (Michelangeli et al., 1995). Ca\(^{2+}\) homeostasis between the ER and cytoplasm is crucial for the proper function of the ER, as Ca\(^{2+}\) is needed to maintain the structure of the ER and ER molecular chaperones require Ca\(^{2+}\) binding for proper protein folding (Stevens and Argon, 1999; Terasaki et al., 1996). Tunicamycin is another ER stress inducer which inhibits oligosaccharyl transferase
(OST), thereby blocking N-linked glycosylation that is required for folding of nascent peptides (Lehle and Tanner, 1976; Schroder and Kaufman, 2005). Similarly, disulfide bonds can be reduced by DTT, resulting in accumulation of misfolded proteins in the ER (Schroder and Kaufman, 2005). Because inducers that cause severe and chronic ER stress can trigger apoptosis, the regulation of the accumulation of unfolded proteins in the ER is crucial for cell survival.

The accumulation of unfolded proteins is sensed via PERK, which phosphorylates eIF2alpha at serine 51, resulting in the attenuation of overall protein synthesis (Harding et al., 1999; Ron and Harding, 2007; Scheuner and Kaufman, 2008). This event is known to carry out two major functions. Firstly, it prevents the accumulation of newly synthesized proteins in the ER. Secondly, it reprograms gene expression through the translation of selective mRNAs. One of these mRNAs is ATF4, which encodes a transcription factor that activates transcription of stress response genes including chaperones and ERAD proteins, antioxidative stress response proteins, and proteins required for amino acid biosynthesis and transport (Figure 1.4) (Harding et al., 2000; Harding et al., 2003; Scheuner et al., 2001; Vattem and Wek, 2004). Most of these stress-induced genes are adaptive, and are thought to help the cell survive during ER stress. However, ATF4 also induces transcription of CHOP (GADD153), which is another transcriptional factor that triggers apoptosis (Scheuner and Kaufman, 2008). Thus, it is likely the balance of adaptive and apoptotic gene expression that determines whether the cell survives or triggers apoptosis. For instance, cells in which CHOP was deleted were unable to trigger apoptosis under ER stress (Fawcett et al., 1999; Zinzsner et al., 1998). CHOP is also important in inducing transcription of GADD34 (Marciniak et al., 2004).
Accumulation of unfolded/misfolded proteins is sensed by three resident proteins: PERK, ATF6, and IRE1. Upon activation, PERK phosphorylates eIF2alpha to inhibit overall translation to reduce the protein load in the ER. At the same time, eIF2alpha phosphorylation leads to upregulation of ATF4, a transcription factor required for expression of various UPR genes. Normally inactive ATF6 undergoes proteolysis in the Golgi Apparatus upon accumulation of unfolded/misfolded proteins, and this produces a transcriptionally active ATF6 fragment that can now upregulate transcription of UPR genes. Activated IRE1 initiates splicing of XBP1 mRNA to activate its translation, and XBP1 transcriptionally upregulates UPR genes. In addition, IRE1 contains RNase activity to degrade mRNAs, again leading to reduction in the ER protein load (Scheuner and Kaufman, 2008 modified by permission).
GADD34 forms a complex with the catalytic subunit of protein phosphatase 1 (PP1) to dephosphorylate eIF2alpha, ultimately leading to recovery of overall translation following ER stress. This recovery in translation allows stress-induced transcripts to be synthesized to counteract the stress (He et al., 1996; Novoa et al., 2001; Novoa et al., 2003). Indeed, more studies into the regulation of pro- and anti-apoptotic signals during ER stress are required.

Another component of the UPR is IRE1alpha which is also activated by dimerization and autophosphorylation in response to accumulation of unfolded proteins in the ER (Ron and Walter, 2007). Activated IRE1alpha acts as an endonuclease that cleaves the mRNA encoding the basic leucine zipper-containing-transcription factor x-box binding protein 1 (XBP1) (Calfon et al., 2002; Lee et al., 2002; Yoshida et al., 2001). Normally, the unspliced XBP1 mRNA encodes for XBP1u, which inhibits the transcription of key UPR genes encoding chaperones and ERAD proteins (Lee et al., 2003; Oda et al., 2006; Yoshida et al., 2001; Yoshida et al., 2006). However, once spliced, the XBP1 mRNA encodes a potent activator of the transcription of these stress-response UPR genes (Figure 1.4) (Lee et al., 2003; Oda et al., 2006; Yoshida et al., 2001). Another function of IRE1alpha is that it has RNase activity to degrade ER localized mRNAs to relieve the load of overall proteins accumulating in the ER (Hollien and Weissman, 2006). Lastly, it has been postulated that phosphorylated IRE1alpha can also bind to tumor necrosis factor receptor (TNFR)-associated factor-2 (TRAF2) to activate caspase-12, leading to eventual cell death (Yoneda et al., 2001).

ATF6, the third component of the ER stress sensors, is normally tethered to the ER membrane as an inactive precursor, and under ER stress, it is activated through 1) release
from the ER chaperone BiP, 2) localization to the Golgi Apparatus from the ER, and 3) subsequent cleavage by Golgi-resident proteases (Haze et al., 1999; Ron and Walter, 2007; Shen et al., 2002). The remaining cytosolic fragment of ATF6 is transported to the nucleus, and induces the transcription of genes for chaperones and ERAD proteins, catalyzing protein folding and degradation (Figure 1.4) (Haze et al., 1999; Wu et al., 2007; Yamamoto et al., 2007).

The importance of the PERK-eIF2alpha pathway in normal cellular homeostasis is highlighted by its defect in numerous physiological complications. For example, ER stress is linked to obesity and type 2 diabetes. In the obese mutant mice (ob/ob), both PERK and eIF2alpha are phosphorylated, which are key ER stress markers, indicating that increased fatty acid levels can induce ER stress (Ozcan et al., 2004). Moreover, ER stress has been found to disrupt the insulin receptor signalling pathway in liver cells (Ozcan et al., 2004). In type 2 diabetes patients, the level of UPR-induced proteins such as CHOP and BiP is also elevated (Laybutt et al., 2007). Defects in the ER stress pathway can also negatively affect glucose homeostasis and insulin signalling (Ozcan et al., 2004). This is exemplified by mutations found within human PERK in Walcott-Rallison syndrome patients. Symptoms of this syndrome also include osteopenia, cardiovascular disease, mental retardation, and diabetes (Delepine et al., 2000; Scheuner and Kaufman, 2008; Senee et al., 2004). Further support that this pathway can lead to pathophysiological defects came through transgenic mice studies. PERK knockout mice exhibit diabetes-like symptoms due to defective functioning of beta-cells and the exocrine pancreas (Zhang et al., 2002). In addition, mice containing an eIF2alpha mutation which is incapable of phosphorylation (Ser51Ala) show beta-cell deficiency and
failure to respond to ER stress (Scheuner et al., 2001). In addition to diabetes, inability to 
elicit UPR and to counteract ER stress was found to result in neuronal death in 
Alzheimer's disease, further emphasizing the importance of the PERK-eIF2alpha 
pathway (Katayama et al., 2004). Given that defects in the PERK-eIF2alpha pathway are 
closely linked to diabetes and other diseases, elucidation of the downstream signalling 
events is important in understanding the progression of these diseases.

1.6 Translational Regulation by Upstream Open Reading Frames

Paradoxically, a subset of mRNAs can preferentially be translated during ER stress 
when overall cap-dependent translation is inhibited. How these mRNAs bypass this 
translational inhibition is an outstanding question in the field. One major mechanism that 
is used by several mRNAs is through upstream open reading frames within the 5’ 
untranslated region (5’ UTR). uORFs are commonly found in mRNAs encoding for 
oncogenes, growth factors, and cellular receptors (Kozak, 1987a). In fact, a recent study 
has revealed that uORFs are found in approximately 50% of human and mouse 
transcripts, and tend to be evolutionarily conserved across different species (Calvo et al., 
2009; Iacono et al., 2005; Zhang and Dietrich, 2005). Despite uORFs’ relatively high 
prevalence, only a handful of uORFs have been studied in detail. In general, uORFs act 
as barriers to scanning ribosomes before the main ORF AUG thereby repressing 
translation (Sachs and Geballe, 2006). However, ribosomes can still access the main ORF 
via a number of ways and under certain conditions. One of them is by leaky scanning of 
ribosomes, as many uORF AUGs do not have an optimal Kozak consensus ([A/G] CC
AUG G), and therefore ribosomes can bypass them during scanning (Kozak, 1984; Sachs and Geballe, 2006; Suzuki et al., 2000).

The classic example of translational regulation via uORF is shown in the *Saccharomyces cerevisiae* *GCN4* mRNA. *GCN4* encodes a transcription factor that activates transcription of amino acid biosynthetic genes, and is vital for yeast to respond to amino acid starvation. *GCN4* is translationally upregulated during amino acid starvation when eIF2alpha is phosphorylated, and this regulation is mediated by four uORFs in its 5' UTR (Hinnebusch, 2005). Basally, after translation of the first uORF, ribosomes reinitiate at the downstream uORFs (Figure 1.5A). After translating uORF4, the ribosomes dissociate from the transcript, so that ribosomes do not reach the main *GCN4* ORF and GCN4 is not synthesized. During amino acid starvation when eIF2alpha is phosphorylated by GCN2, the pool of the TC allows ribosomes to reach the *GCN4* ORF. In this scenario, after translating uORF1, ribosomes resume scanning, and approximately 50% of the ribosomes will bypass uORF4 before picking up another TC to initiate translation at the *GCN4* AUG (Figure 1.5A) (Hinnebusch, 2005; Hinnebusch et al., 2007). It has been shown that the C+G-rich sequences surrounding the uORF4 stop codon stimulate translation termination whereas recent studies have shown that resumption of scanning after *GCN4* uORF1 translation is dependent on interaction between yeast eIF3 and the “enhancer” sequence at the 5’ of *GCN4* uORF1 (Grant and Hinnebusch, 1994; Szamecz et al., 2008). Normally, the reinitiation mechanism depends on several properties. One is the spacing between upstream and downstream ORFs. The longer the spacing, the greater the chance for the scanning ribosome to recruit the TC and initiate at the downstream AUG of the uORF (Kozak, 1987b). In contrast, the shorter the
Figure 1.5  Translational Regulation via Upstream Open Reading Frames (uORFs)

(A) Translation of *GCN4* is upregulated via its uORFs when eIF2alpha is phosphorylated during amino acid starvation, as approximately 50% of scanning ribosomes can now reinitiate at *GCN4* AUG after uORF1 translation due to TC scarcity (Hinnebusch, 2005 modified by permission).

(B) When *CAT-1* uORF is not translated, its IRES structure is masked (Stage I). Once *CAT-1* uORF gets translated, conformational changes expose an IRES that can now recruit ribosomes and initiate translation during amino acid starvation (Stages II and III) (Yaman et al., 2003 modified by permission).

(C) At low [Arg], the ribosomes can reach *CPA1* AUG past uORF by leaky scanning, and translate *CPA1* ORF. At high [Arg], AAP from uORF stalls the ribosome at its termination codon, preventing access of the ribosomes to *CPA1* AUG.
spacing, the higher the chance for the ribosomes to bypass the downstream uORF, and initiate translation at the main ORF AUG (Kozak, 1987b). Also, the length of the uORF affects reinitiation efficiency, as reinitiation becomes less efficient with an increasingly longer uORF (Kozak, 2001; Rajkowitsch et al., 2004). However, there are examples of exceptional reinitiation mechanisms after ribosomes translate a long ORF. For instance, ribosomes that translate an upstream ORF in feline calicivirus (FCV) genome can efficiently reinitiate at a downstream cistron (Poyry et al., 2007). In this example, reinitiation is mediated by 3’ elements that can recruit the eIF3/40S complexes (Poyry et al., 2007). This phenomenon is not observed in eukaryotes in which only reinitiation after translation of a relatively short ORF is permitted because retention of eIF4G appears to be required for reinitiation (Kozak, 2001; Poyry et al., 2004).

The reinitiation mechanism used by GCN4 may be a general mechanism to regulate other mRNAs. ATF4, a transcription factor that is translated during ER stress when eIF2alpha is phosphorylated, contains two uORFs that mediate translation in a manner similar to the GCN4 mRNA. Specifically, the upstream uORF of ATF4 stimulates reinitiation of the ribosomes, while the downstream uORF overlaps with the ATF4 ORF and is thus inhibitory (Lu et al., 2004). Recently, it has also been shown that ATF5 may also be regulated similarly (Zhou et al., 2008). Given that uORFs are present in ~50% of all mRNAs, it remains to be seen how prevalent this mechanism is in regulating other mRNAs.

uORFs can also modulate RNA structure to affect translation. One interesting example is the uORF of the eukaryotic cationic amino acid transporter-1 (CAT-1) mRNA. CAT-1 translation is upregulated during amino acid starvation (Hyatt et al., 1997). This
regulation is mediated by a single uORF in its 5’ UTR. Translation of this 48 amino acid long CAT-1 uORF leads to structural changes in the mRNA to form an IRES element, which is an RNA element that can directly recruit ribosomes in a cap-independent manner (Yaman et al., 2003). It is thought that stimulation of this IRES also requires an IRES trans-acting factor (ITAF), which is synthesized during amino acid starvation (Figure 1.5B) (Fernandez et al., 2005; Yaman et al., 2003). Under basal conditions, uORF translation acts a barrier to block ribosomes from reaching the CAT-1 AUG (Figure 1.5B) (Yaman et al., 2003).

The amino acid sequence of select uORFs has also been found important for regulating translation. One prominent example is Saccharomyces cerevisiae CPA1 mRNA. CPA1 encodes the carbamoylphosphate synthetase, which is an enzyme required for arginine biosynthesis (Thuriaux et al., 1972). CPA1 expression is induced when arginine levels are low, and its expression is repressed when arginine levels are high (Werner et al., 1987). Regulation of CPA1 translation is mediated by a uORF, which encodes the arginine attenuator peptide (AAP). Under conditions when arginine levels are low, approximately 50% of ribosomes will scan past the AAP uORF and initiate translation of the main CPA1 ORF. This is because the AUG codon of the uORF is in a sub-optimal Kozak context. The other 50% of the ribosomes will translate the uORF, terminate, and dissociate from the mRNA. When arginine levels are high, ribosomes that translate the uORF will stall in an amino acid sequence- and arginine-dependent manner (Werner et al., 1987). Thus, the stalled ribosome will consequently block all ribosomes from scanning and inhibit CPA1 translation (Figure 1.5C) (Fang et al., 2004; Gaba et al., 2001). Recently, it has been shown that increased occupancy of ribosomes on the CPA1
uORF through arginine-induced ribosome stalling at its termination codon also triggers nonsense mediated decay (NMD) (Gaba et al., 2005; Jacobson and Izaurralde, 2007). Thus, the multiple mechanisms that are regulated by the uORF highlight the importance of controlling CPA1 mRNA in response to arginine levels.

1.7 GADD34

Growth arrest and DNA damage-inducible gene 34 (GADD34) is an important gene involved in the UPR. GADD34 was originally discovered as a gene that was induced in response to various genotoxic stresses. The first clue to the function of GADD34 came from studies of the \( \gamma_1 \)34.5 gene of herpes simplex virus 1 (HSV-1). \( \gamma_1 \)34.5 expression prevented the inhibition of host protein synthesis by interacting with protein phosphatase type 1 (PP1) to dephosphorylate eIF2alpha (Chou and Roizman, 1992; Chou and Roizman, 1994; He et al., 1997; Zhan et al., 1994). Thus, \( \gamma_1 \)34.5 expression is important in counteracting specific host antiviral responses such as the activation of PKR and phosphorylation of eIF2alpha, thus allowing translation of herpes virus (He et al., 1997). Subsequent studies showed that the carboxy-terminal domain of HSV-1 \( \gamma_1 \)34.5, which is the region that interacts with PP1, is similar to the corresponding region of GADD34. Studies of protein chimera showed that their carboxy-terminal domains are functionally interchangeable, strongly suggesting that GADD34’s role is to dephosphorylate eIF2alpha (Chou and Roizman, 1994; He et al., 1996; He et al., 1997). Indeed, GADD34 has been shown to bind PP1 to dephosphorylate eIF2alpha following PERK activation during ER stress. This eventually leads to recovery of overall protein synthesis (Figure 1.6) (He et
al., 1996; Novoa et al., 2003). In cells containing a mutant GADD34 that lacks its carboxy-terminal domain, eIF2alpha remained phosphorylated during ER stress, and overall translation recovery was not observed (Novoa et al., 2003). These mutant cells are sensitized to ER stress, and cannot survive ER stress as well as wild-type cells. It has been proposed that recovery of protein synthesis allows the translation of stress-induced mRNAs during ER stress. Therefore, increased expression of GADD34 during eIF2alpha phosphorylation acts in a negative feedback loop of the ER stress pathway in order for cells to survive (Figure 1.6) (Novoa et al., 2003).

Under ER stress, GADD34 transcription is upregulated by transcription factors ATF4 and CHOP. Binding sites for these transcription factors have been identified in the promoters of genes (Ma and Hendershot, 2003; Novoa et al., 2001). Although the transcriptional activation mechanism of GADD34 has been well established, how its transcripts are translated to produce GADD34 protein during ER stress when overall translation is inhibited by eIF2alpha phosphorylation is still not well understood. Interestingly, human and mouse GADD34 contains two uORFs in its 5' UTR. My thesis focuses on elucidating the role of these uORFs on GADD34 translation during ER stress.

1.8 Thesis Investigation

Although transcriptional activation of GADD34 via ATF4 and CHOP in the ER stress pathway has been well studied, the key question remains: How can GADD34 transcripts bypass the translational block by eIF2alpha phosphorylation? It is possible that that the
Upon ER stress, PERK is activated to phosphorylate eIF2alpha. This leads to inhibition of overall protein synthesis, but also activates translation of ATF4 mRNA. ATF4, which encodes a transcription factor, induces transcription of CHOP, another transcription factor that activates proapoptotic genes. GADD34 transcription is known to be induced by both CHOP and ATF4. GADD34 binds to protein phosphatase I (PP1) complex to dephosphorylate eIF2alpha, leading to recovery of overall protein synthesis. The mechanism by which GADD34 mRNA is translated during eIF2alpha phosphorylation is still unknown, and is the main research question of this thesis.
increase in \textit{GADD34} mRNA levels is sufficient to allow high enough expression of \textit{GADD34}, and thereby initiate the negative feedback loop in the ER stress response pathway. Alternatively, \textit{GADD34} may be translationally upregulated to maximally produce GADD34 during ER stress. My hypothesis is that translational regulation of \textit{GADD34} via its 5’ UTR uORFs allows optimal expression of \textit{GADD34} in order to recover protein synthesis during ER stress. The results presented in this thesis identify a unique mechanism by which 5’ UTR uORFs regulate expression of \textit{GADD34} during eIF2alpha phosphorylation, thereby providing another complexity in the regulation of the ER stress response pathway. In addition, this work may provide insight into and may serve as a model for understanding how other uORF-containing mRNAs may be regulated.

Chapter two of this thesis addresses whether \textit{GADD34} is translationally regulated. My work shows that \textit{GADD34} mRNA is translated during ER stress even when its transcription is blocked by the transcriptional inhibitor, actinomycin D. Moreover, we show specifically that the presence of the downstream uORFs is important for this regulation. These results have been published in the Journal of Biological Chemistry in 2009 (Lee et al., 2009).

Chapter three of this thesis investigates the mechanism of \textit{GADD34} translation by the uORFs in greater detail. I have found that the \textit{GADD34} uORF2 and the intercistronic space are sufficient to regulate translation of a reporter RNA during eIF2alpha phosphorylation. In addition, various mutants of \textit{GADD34} uORF2 were tested for their effect on translational regulation, providing insight into the unique mechanism of \textit{GADD34} translation.
Chapter four illustrates the development of *in vitro* systems that recapitulate *in vivo* conditions when translation is compromised. These include inhibition of eIF2 activity and eIF4F complex formation. The development of such systems could prove to be extremely useful in determining the position of scanning ribosomes on the *GADD34* 5' UTR. Some of the results from this were published in the Journal of Virology in 2009, of which I am a co-author (Garrey et al., 2009).

Lastly, chapter five summarizes and discusses the research findings and suggests future directions for the studies. All studies were carried out by me except for the sucrose density gradient experiments with human HepG2 and mouse Hepa cells and the immunoprecipitation of newly synthesized proteins that are radiolabelled *in vivo* (Figures 2.1, 2.2, 2.8, 2.10) (Dr. E. Jan).
CHAPTER II
Translational Regulation of GADD34 Through 5’ UTR uORFs during eIF2alpha Phosphorylation

2.1 Introduction

2.1.1 Translational Regulation of GADD34 during ER Stress

Several lines of evidence from previous work in the lab indicate that GADD34 is translationally regulated during ER stress. In human HepG2 and mouse Hepa cells, induction of ER stress by thapsigargin or DTT treatment inhibits protein synthesis and induces eIF2alpha phosphorylation (Figure 2.1). Similarly, translationally active polysomes are greatly reduced during ER stress (Figure 2.2A), indicating that the majority of mRNAs is translationally repressed. However, GADD34 mRNAs are associated with more ribosomes, and are shifted to higher molecular weight fractions during thapsigargin-induced and DTT-induced ER stress compared to basal conditions (Figure 2.2B-E). This indicates that GADD34 mRNAs are actively translated during ER stress. Similarly, ATF4 mRNAs are also shifted to higher molecular weight fractions during ER stress, in support of previous data that ATF4 is translationally upregulated during ER stress (Figure 2.2B and C). In contrast, mRNAs such as human Actin and mouse GADPH, which typify the majority of mRNAs in the cell, are associated with fewer ribosomes under ER stress, indicating that these mRNAs are translationally repressed during ER stress (Figure 2.2B-E). These data suggest that GADD34 is
Figure 2.1  Overall Translation Inhibition and eIF2alpha Phosphorylation by ER stress Inducing Agents in HepG2 and Hepa Cells

(A) $[^{35}\text{S}]$methionine/cysteine pulse-labeling of total proteins in HepG2 cells treated with either 1μM thapsigargin or 2 mM DTT over 60 minutes (top), and Western blots of total and phosphorylated eIF2alpha from the same experiment (bottom).

(B) $[^{35}\text{S}]$methionine/cysteine pulse-labeling of total proteins in Hepa cells treated with 1μM thapsigargin for 30 minutes (top), and Western blots of total and phosphorylated eIF2alpha from the same experiment (bottom). All figures courtesy of Dr. E. Jan.
Figure 2.2  Polysomal Association of GADD34 mRNA during eIF2alpha Phosphorylation in HepG2 and Hepa Cells

(A) Polysome profiles of HepG2 cells that are mock-treated (left) or treated with 1 μM thapsigargin for 30 minutes (right).

(B) Northern blots of selected endogenous RNAs collected from polysomal analysis of HepG2 cell lysates after 1 μM thapsigargin treatment for 30 minutes. The fractions containing a single (80S) and multiple ribosomes (polysomes) are indicated below.

(C) Northern blots of select endogenous RNAs collected from polysomal analysis on Hepa cell lysates after 1 μM thapsigargin treatment for 30 minutes.

(D) Quantification of the Northern blots from (B). The amount of a radioactive probe is plotted as a percentage of the signal in all fractions within a single sucrose gradient.

(E) Quantification of the Northern blots from (C). The amount of a radioactive probe is plotted as a percentage of the signal in all fractions within a single sucrose gradient. All figures courtesy of Dr. E. Jan.
translationally active during ER stress, and this thesis focuses on the mechanism of this regulation.

2.2 Materials and Methods

2.2.1 Antibodies Used in This Study

Polyclonal antibody to CHOP (Affinity Bioreagents, MAI-250) was a generous gift of Dr. Jim Johnson, University of British Columbia, Canada. Other polyclonal antibodies used for Western blotting and immunoprecipitation were as follows: total eIF2alpha Antibody (Cell Signaling, #9722) to the C-terminal segment of eIF2 alpha; phosphor-eIF2alpha Antibody (Cell Signaling, #9721); GADD34 Antibody (Santa Cruz Biotechnology, H-193), ATF4 Antibody (Santa Cruz Biotechnology, SC-200), and GFP Antibody (Roche Applied Science), which cross-reacts with YFP.

2.2.2 Cell Culture and Stable Cell Lines

HepG2 cells were grown in Dulbecco’s modified Eagle medium (DMEM) (Sigma) with 2 mM L-glutamine (Invitrogen), 50 µg/ml penicillin/streptomycin (Invitrogen), and 10% fetal bovine serum (v/v) (HiClone). Stable cell lines were created by transfecting reporter constructs using Lipofectamine 2000 (Invitrogen) at approximately 50% confluency, and selecting with 1 mg/ml Geneticin (Invitrogen) for two to three weeks. Finally, the selected cell lines were pooled together.
2.2.3 In vivo Radiolabel Incorporation of Newly Synthesized Proteins

In order to detect newly synthesized proteins, cells were incubated in DMEM minus methionine supplemented with 10% (v/v) dialyzed fetal bovine serum (FBS) at 37 °C for 25 minutes, and metabolically labelled with 100 μCi/ml [35S]methionine/cysteine (Perkin Elmer) at 37 °C for 20 minutes. Cellular stresses were induced by adding 1 μM thapsigargin (Sigma), 2 mM DTT, or 100 μg/ml arsenite (Riedel de Haen). Subsequently, radiolabeled cells were washed twice with 1 X PBS, and collected in the lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5% (v/v) Nonidet P-40, and protease inhibitor mixture (Roche Applied Science). Bradford reagent (Bio-Rad) was used to determine the concentrations of the proteins collected.

2.2.4 SDS-PAGE and Western Blot Analysis

Cells were washed twice with PBS with 10 mM NaF and 17.5 mM β-glycerophosphate, and scraped into lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100 (v/v), 10% glycerol, 1 mM EDTA, 10 mM NaF, 17.5 mM β-glycerophosphate, and a protease inhibitor mixture (Roche Applied Science). The lysates were freeze-thawed three times before they were centrifuged at 10,000 rpm for 10 minutes at 4 °C to clear cell debris and nuclei. Bradford reagent (Bio-Rad) was used to determine the protein concentration. For Western blotting, protein samples were denatured in 2 X SDS loading dye at 95 °C for 5 minutes, and separated on 8% or 12% SDS polyacrylamide gels. Proteins were subsequently transferred onto a PVDF
Immобilon-P (Millipore) or nitrocellulose Trans-Blot Transfer Membrane (Bio-Rad). The membranes were blocked in 5% skim milk in TBS containing 0.1% Tween 20 (TBS-T) for 30 minutes at RT, and incubated in the primary antibody (1:1000 dilution) in 1% or 5% BSA in TBS-T overnight at 4 °C. The blots were washed three times with TBS-T, and probed with a secondary anti-mouse or anti-rabbit antibody conjugated to LI-COR IRDye 680/800 (1:10,000) (Rockland, Gilvertsville, PA) or to HRP (1:10,000) (Amersham) in 5% skim milk in TBS-T for 1 hour at RT. The proteins were finally detected by a LI-COR Odyssey imager (Lincoln, NE) or ECL (Millipore).

2.2.5 Sucrose Density Gradient Centrifugation and Polysome Analysis

Fresh medium was added to cells 4.5 hours prior to 1 µM thapsigargin treatment. Following drug treatment for 30 minutes at 37 °C, the cells were incubated with 100 µg/ml cycloheximide for 3 minutes at 37 °C. Subsequently, the cells were washed three times with 1 X PBS containing 100 µg/ml cycloheximide, and lysed with buffer containing 15 mM Tris-HCl (pH 7.4), 15 mM MgCl2, 200mM NaCl, 1% Triton X-100 (v/v), 100 µg/ml cycloheximide, and 1 mg/ml heparin. To clear cell debris and nuclei, the lysates were centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was loaded onto a 10-50% (w/v) sucrose gradient containing lysis buffer without Triton X-100. The gradient was centrifuged at 35,000 rpm for 2 hours 45 minutes to 3 hours in an SW41 rotor (Beckman Coulter) at 4 °C. The gradient was fractionated by using Teledyne ISCO fraction collector and a Brandel syringe pump system. The fractions were mixed with 3 ml of 8 M guanidine-HCl and 5 ml of 100% EtOH to isolate RNA by precipitation.
at -80 °C overnight. The RNA pellet was resuspended in water and precipitated again in 100% EtOH and 3 M NaOAc (pH 5.2) at -20 °C overnight. The RNA pellet was finally washed with 75% EtOH and resuspended in water. Equal volumes of RNA then were subjected to Northern blot analysis.

2.2.6 Northern Blot Analysis

Total RNA from cells was purified using TRIzol (Invitrogen) following treatment with drugs. The total RNA and RNA purified from sucrose gradient centrifugation fractions were separated on a 1% denaturing RNA gel (1 X MOPS and 20% Formaldehyde). RNAs on the gel were transferred to a Zeta-probe membrane (Bio-Rad) at RT overnight. The RNAs were UV-crosslinked to the membrane, and the transfer of RNA was confirmed by methylene-blue staining. The membrane was pre-hybridized with 5 ml Pre-hybridization Buffer (0.5 M NaPO₄, 10 mM EDTA, 7% Sodium dodecyl sulfate (SDS)) at 65 °C for at least 15 minutes. [α-³²P] dATP labelled DNA probe at one million counts per ml was generated using the Radprime kit (Invitrogen) or DecaLabel DNA Labeling kit (Fermentas). The probes were heated at 95 °C for 5 minutes prior to incubation at 65 °C overnight with the pre-hybridized membrane. The membrane was washed at least three times with 15 ml of Wash Buffer (40 mM Tris, pH 7.5, 1 mM EDTA, 0.5% SDS) at 65 °C for minimum 15 minutes before it was exposed under a phosphor screen. The phosphor screen was scanned by a Typhoon Imager (GE Healthcare) the next day, and the radioactivity was quantified using ImageQuant software (GE Healthcare).
2.3 Results

2.3.1 Transcriptional and Translational Regulation of GADD34 under ER Stress

In addition to its transcriptional activation, GADD34 is translationally regulated under ER stress by polysome analysis (Ma and Hendershot, 2003; Novoa et al., 2001) (Figure 2.2). To determine the transcriptional and translational contributions to GADD34 expression during ER stress, we wished to uncouple these contributions to GADD34 induction. Toward this, HepG2 cells were treated with 5 µg/ml actinomycin D, a transcriptional inhibitor in the presence or absence of 1 µM thapsigargin for 3 or 6 hours (Figure 2.3 and 2.4). Cells were harvested, and total RNA and protein lysates were collected. Northern blot analysis indicated that levels of GADD34, ATF4, and CHOP mRNAs increased upon treatment of thapsigargin for 3 and 6 hours, confirming previous reports that these mRNAs are transcriptionally induced during ER stress. In contrast, GADPH mRNA levels were repressed under the same conditions (Figure 2.3A and B). As expected, the transcriptional upregulation of these ER stress-induced mRNAs were inhibited upon actinomycin D treatment in the presence or absence of thapsigargin (Figure 2.3A and B).

We then examined the protein expression of these ER stress markers under the same conditions. As expected, the level of GADD34 protein increased after 3 hours thapsigargin treatment, and by 6 hours, maximal expression of GADD34 protein was achieved (Figure 2.4A and B). Thapsigargin treatment, but not actinomycin D alone, induced eIF2alpha phosphorylation after 3 hours of treatment (Figure 2.4A). GADD34
Figure 2.3  Transcriptional Activation of UPR mRNAs under ER Stress

(A) Northern blots (see Materials and Methods 2.2.6) of mRNAs from HepG2 cells that were treated with 5 μg/ml actinomycin D (actD) and/or 1 μM thapsigargin (thap) for 3 or 6 hours.

(B) Quantification of the Northern blots from (A). The amount of each transcript is measured as a fold increase of signal from the 3 hours mock-treated sample.
Figure 2.4  Translational Upregulation of UPR Genes during ER Stress-Induced eIF2alpha Phosphorylation

(A) Western blots (see Materials and Methods 2.2.4) of proteins from HepG2 cells that are treated with 5 µg/ml actinomycin D (actD) and/or 1 µM thapsigargin (thap) for 3 or 6 hours. For GADD34, two independent experiments with reproducible induction of GADD34 are shown. In the top panel, the arrow indicates GADD34 protein, and the asterisk represents the unspecific protein band detected by the GADD34 antibody.

(B) Quantification of the Western blot of the representative GADD34 from (A). The GADD34 protein expression was measured by LI-COR Odyssey imaging system (Lincoln, NE), and the value of each sample was normalized to that of a 3 hours mock-treated sample.
induction led to the decrease of eIF2alpha phosphorylation at later times during ER stress (6 hours) (Figure 2.4A), which is in agreement with the previous finding that the decrease in eIF2alpha phosphorylation is due to GADD34-mediated eIF2alpha dephosphorylation, and coincides with recovery of overall protein synthesis (Novoa et al., 2003). As shown previously, ATF4 and CHOP protein levels were also induced during thapsigargin treatment (Figure 2.4A). When the cells were treated with both thapsigargin and actinomycin D, CHOP protein was not detected, indicating that expression of CHOP is dependent on transcriptional induction (Figure 2.4A). In contrast, ATF4 protein was still detected under this condition, yet to a lower extent than after thapsigargin treatment alone (Figure 2.4A). This confirms that ATF4 is translationally upregulated, and the increase is likely due to existing ATF4 mRNA being translationally induced via its uORFs. This also indicated that the maximal ATF4 expression is mediated by both translational and transcriptional controls (Figure 2.4). Interestingly, eIF2alpha remained phosphorylated after 6 hours treatment of actinomycin D and thapsigargin, as the induction of GADD34 was not as prominent as during thapsigargin alone at that time point (Figure 2.4A and B). This suggested that both transcriptional and translational activation are required to achieve maximum expression of GADD34 to permit dephosphorylation of eIF2alpha at the later stages of ER stress. To demonstrate that expression of GADD34 protein during actinomycin D/thapsigargin treatment was due to translational upregulation of the pre-existing GADD34 mRNA, its polysomal association was monitored (Figure 2.5). Indeed, both treatments (thapsigargin alone and actinomycin D/thapsigargin) elicited a shift of GADD34 mRNAs to higher molecular weight polysomal fractions as compared with that
Figure 2.5  Polysomal Association of mRNAs after ER Stress and Inhibition of Transcription by Actinomycin D

Northern blots of human GADD34 and GAPDH mRNAs collected from polysomal analysis on HepG2 cell lysates after 1 µM thapsigargin treatment (thap), 1 µM thapsigargin and 5 µg/ml actinomycin D treatment (actD/thap), or mock-treatment (unt) for 30 minutes.
in untreated cells (from fraction #7 to #8) (Figure 2.5), indicating that during ER stress, 
\textit{GADD34} mRNA is actively translated to induce GADD34 protein expression.

\[ \text{2.3.2 Polysomal Association of } GADD34 \text{ mRNA via its } 5' \text{ UTR under ER Stress} \]

The preceding experiments indicated that \textit{GADD34} is translationally regulated 
during ER stress. To begin probing the translational mechanism of \textit{GADD34}, the 
organization of the 5' UTRs of different mammalian \textit{GADD34} was analyzed (Figure 2.6). 
We focused on the 5' UTRs because these regions often play a key role in regulation of 
gene expression (van der Velden and Thomas, 1999). The 5' UTRs of \textit{GADD34} from 
select mammalian species ranged from 207 to 269 nucleotides, and all contained two 
uORFs (namely uORF1 and uORF2 for upstream and downstream uORFs, respectively) 
(Figure 2.6). Human and rat \textit{GADD34} uORFs are separated by 30 and 7 nucleotides, 
respectively, whereas the hamster and mouse \textit{GADD34} uORFs overlap each other by 
one nucleotide such that the AUG start codon of the downstream uORF overlaps the 
UGA stop codon of the upstream uORF (Figure 2.6). In order to determine if the 
\textit{GADD34} 5' UTR plays a regulatory role on its translation during ER stress, reporter 
constructs were engineered to contain the 5' UTR of the human or mouse \textit{GADD34} and 
\textit{YFP} ORF under the transcriptional control of the viral CMV promoter. Stably expressing 
HepG2 cell lines were then selected (Figure 2.7).
Figure 2.6  uORFs in the 5' UTR of the GADD34 Genes from Different Mammalian Species

The schematic illustrates 5' UTR of GADD34 from human (NM_014330), rat (NM_133546), hamster (L28147), and mouse (NM_008654) obtained from GenBank™. The number of amino acids in each box indicates the sizes of two uORFs present in this region, and AUG of the main GADD34 ORF is also shown. uORFs in hamster and mouse GADD34 5' UTR overlap each other, and this overlap in mouse GADD34 uORFs is depicted at nucleotide level (below).
Figure 2.7  Polysomal Association of Endogenous and YFP Reporter mRNAs during ER Stress-Induced eIF2alpha Phosphorylation

Northern blots of endogenous mRNAs and YFP reporter RNA containing the 5' UTR of GADD34 collected from polysomal analysis on HepG2 cell lysates after mock-treatment (unt) or 1 µM thapsigargin treatment for 30 minutes. The 5' UTR of human GADD34 (A), mouse GADD34 (B), or human ATF4 (D) was fused a YFP reporter construct, and these reporters were stably transfected in HepG2 cells that were subjected to polysomal analysis. Their transcription was under a strong CMV promoter. The reporter containing mutations in the AUGs of two uORFs in human GADD34 5' UTR (B) was also created to address the importance for translational regulation under ER stress.
We first tested whether these reporter mRNAs were associated with polysomes during ER stress. Following the thapsigargin treatment for 30 minutes, the cell extracts were subjected to sucrose density gradient centrifugation to monitor polysomal distributions of select mRNAs by Northern blot analysis. Human Actin and GAPDH mRNAs were associated with actively translating polysomes in the untreated cells, but upon thapsigargin treatment, they shifted to lower molecular weight polysomal fractions (Figure 2.7). In untreated cells, endogenous GADD34 and ATF4 mRNAs were mostly found in fractions #6 and #7 (Figure 2.7A). During thapsigargin treatment, these mRNAs sedimented to fractions #7 and #8, indicating that they were translationally upregulated during ER stress (Figure 2.7A). As a positive control, hATF4-YFP reporter mRNAs also shifted to higher molecular weight fractions during ER stress, showing that the human ATF4 5' UTR contains elements crucial for translation during eIF2alpha phosphorylation (Figure 2.7D) (Harding et al., 2000; Lu et al., 2004). Similarly, the hGADD34-YFP and mGADD34-YFP reporters containing the 5’ UTR of human or mouse GADD34 shifted to heavier molecular weight fractions (from fractions #6/7 to 8) during ER stress as compared to that in untreated cells (Figures 2.7A and C). This shift to heavier molecular weight fractions of the reporter is similar to that of the endogenous GADD34 mRNA, strongly suggesting that the 5’ UTR contains all of the properties for translational regulation of GADD34 during ER stress (Figure 2.7A and C).

To test if the uORFs are important for this regulation, we mutated the AUG codons of two uORFs in human GADD34 5’ UTR to AUU (1&2 KO). Interestingly, the YFP reporter RNA bearing this mutation sedimented to heavier molecular weight polysomal fractions compared to the WT reporter RNA in the untreated cells (Figure 2.7A and B).
This result indicated that the uORFs are important in repressing translation basally. Upon thapsigargin treatment, this reporter mRNA associated with fewer ribosomes during ER stress, indicating that the mRNA lost the ability to regulate translation without the uORFs (Figure 2.7A and B). In summary, these results suggest that the uORFs of GADD34 5’ UTR basally repress the translation of the transcript, yet are required to mediate its translational upregulation during eIF2alpha phosphorylation.

2.3.3 GADD34 uORFs-Mediated Translational Regulation under ER Stress

Although GADD34 mRNAs were found to be associated with a higher molecular weight polysomal complex under ER stress, this does not prove that protein is actually synthesized. In order to determine whether GADD34 mRNA is indeed associated with translating ribosomes, incorporation of [35S]methionine/cysteine into newly synthesized YFP was directly measured by pulse labeling, followed by immunoprecipitation in the cell lines expressing GADD34 5’ UTR reporters. Using this approach, the role of the two uORFs in the mouse GADD34 5’ UTR was investigated. The uORFs in mouse GADD34 5’ UTR are arranged in a distinctive manner that unlike their human counterparts, overlap each other by one nucleotide (Figure 2.6). For the experiment in Figure 2.8, the upstream uORF (uORF1) and/or the downstream uORF (uORF2) were knocked-out by site-directed mutagenesis. Basal translation of the mGADD34-YFP reporter RNA was repressed, while translation of the mutant 1&2 KO mGADD34-YFP was no longer repressed (Figure 2.8). After thapsigargin treatment, translation of the mGADD34-YFP was unaffected, yet this resistance was lost when both uORFs of mouse GADD34 5’
Figure 2.8 Translational Regulation during eIF2alpha Phosphorylation by the Overlapping uORFs of Mouse GADD34 5' UTR

$[^{35}\text{S}]$methionine/cysteine pulse labeling of overall proteins and immunoprecipitation of YFP protein from HepG2 cells expressing YFP reporter RNAs containing wild-type and mutant mouse GADD34 5' UTR under eIF2alpha phosphorylation. HepG2 cells stably expressing various YFP reporters were either mock-treated (-) or treated with 1 µM thapsigargin (+) to induce eIF2alpha phosphorylation for 45 minutes before overall proteins were pulse labelled with $[^{35}\text{S}]$methionine/cysteine for 20 minutes. SDS-PAGE followed by autoradiography was used to detect immunoprecipitated YFP (first row) and inhibition of overall translation (third row), while eIF2alpha phosphorylation was determined by Western blotting (second row). Total RNA from the cells was also collected to detect the YFP and GAPDH mRNA levels by Northern blotting (fourth row). Figure courtesy of Dr. E. Jan.
UTR were knocked out (Figure 2.8). Moreover, when uORF1 was knocked out while retaining an intact uORF2 (1KO), basal YFP expression was not significantly affected, and like the WT, this reporter RNA was translated during thapsigargin treatment (Figure 2.8). However, when uORF2 was knocked out (2 KO mutant), basal YFP expression was no longer repressed, and thapsigargin treatment inhibited the expression of YFP, exhibiting a similar phenotype as in the 1&2 KO mutant (Figure 2.8). The reporter RNA levels in this experiment were measured by Northern blot analysis, and found to be unaltered by thapsigargin treatment (Figure 2.8). Overall, the data suggest that uORF2 of mouse GADD34 5’ UTR plays a key role in its translational regulation both at the basal level and during eIF2alpha phosphorylation. The uORFs in the human GADD34 5’ UTR were tested by the same approach, and similar results were observed, confirming the conserved regulatory role of the GADD34 uORF2 (Lee et al., 2009).

The previous polysomal analysis has shown that during thapsigargin treatment, hGADD34-YFP and mGADD34-YFP mRNAs were shifted to the heavier molecular weight polysomal fractions, indicating that more of the corresponding proteins should be synthesized under such condition (Figure 2.7). However, it was puzzling to find that the reporter RNAs did not incorporate more radiolabel during ER stress when compared with basal conditions, which is contrary to their polysome distribution (Figures 2.7 and 2.8). This may reflect differences in protocols between the two experiments. In the 
$[^{35}S]$methionine/cysteine labeling experiment, cells were amino acid-starved prior to labeling, whereas the cells were not in the polysome analysis protocol. Therefore, it was possible that starvation of cells prior to thapsigargin treatment could affect translation of the reporter RNAs. In order to test this hypothesis, polysomal analysis of the stable
HepG2 cell line expressing the mGADD34-YFP reporter was performed under amino acid starvation. Indeed, while endogenous GAPDH mRNAs were shifted to the lower molecular weight fractions upon thapsigargin treatment (Figure 2.7), the endogenous GADD34 and the mGADD34-YFP reporter RNAs did not shift, indicating that translational activity of these RNAs is not necessarily enhanced, but maintained under this condition (Figure 2.9A and B). Hence, although its mechanism is unclear, amino acid-starvation was found to disrupt the translational activation via the 5’ UTR of GADD34 under ER stress.

2.3.4 Translation of the GADD34 uORFs

Data so far suggested that the downstream uORF2 of GADD34 is important in translational regulation, as it plays an inhibitory role to keep basal expression of the main ORF low. Moreover, the uORF2 is required to stimulate the main ORF translation under eIF2alpha phosphorylation. Our data show that the uORF1 plays a negligible role. To better understand the roles of the uORFs in this regulation, we determined recognition of the AUGs of these uORFs by the ribosomes.

Here, we engineered mutant mGADD34-YFP reporter RNAs in which the stop codon for each uORF was mutated, and the uORFs were fused in-frame with the reporter YFP ORF (Figure 2.10). For the mGADD34 uORF1-YFP, the AUG codon of uORF2 was mutated to fuse uORF1 in-frame with the YFP ORF. The [35S]methionine/cysteine labeling and immunoprecipitation of YFP from the stable cell lines expressing the mutant reporters revealed that the ribosomes efficiently initiated at the uORF2 AUG (Figure
Figure 2.9  Effect of Starvation on Polysomal Association of mRNAs during ER Stress-Induced eIF2alpha Phosphorylation

(A) Northern blots of select mRNAs collected from polysomal analysis on lysates of HepG2 cell stably expressing a YFP reporter containing the 5' UTR of mouse GADD34. To determine effects of amino acid starvation on translational regulation of GADD34, the cells were incubated in starving media in the presence (st/thap) or absence (st) of 1 μM thapsigargin treatment for 30 minutes.

(B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage of total radioactivity in all fractions within a single sucrose gradient.
Either upstream uORF (uORF1) or downstream uORF (uORF2) of mouse *GADD34* 5' UTR was fused to the downstream *YFP* ORF in frame by mutating its stop codon, and the mutant reporter constructs were stably expressed in HepG2 cells along with the 1&2KO mutant. YFP proteins were immunoprecipitated following $[^{35}S]$methionine/cysteine pulse labeling, and were separated and visualized by SDS-PAGE and autoradiography, respectively. Figure courtesy of Dr. E. Jan.

**Figure 2.10** Leaky Scanning by Ribosomes at the AUG of Mouse *GADD34* 5' UTR Upstream uORF (uORF1)
2.10 Lanes 3 and 4). Interestingly, the uORF1 AUG was not recognized by the ribosomes which seem to bypass the uORF1 and initiate at the downstream AUG instead (Figure 2.10 Lanes 1 and 2).

The nucleotide sequence analysis revealed that the AUGs of both uORF1 and 2 of GADD34 5' UTR are not in the optimal context (Kozak, 1986). As a result, the ribosomes may bypass these non-optimal AUGs through leaky scanning (Kozak, 1991).

The above results suggest that ribosomes bypass the uORF1 AUG to initiate at the uORF2 AUG. To test this further, we strengthened the AUG codon of the uORF1 of the mGADD34 uORF1-YFP (Figure 2.11). By Western blot analysis, it was shown that most ribosomes now initiated translation at the strengthened AUG. This indicated that scanning ribosomes bypass the uORF1 AUG by leaky scanning due to lack of an optimal Kozak consensus sequence (Figure 2.11).

2.4 Discussion

2.4.1 Regulation of GADD34 Expression under ER Stress

eIF2alpha phosphorylation is a major cellular response during ER stress. It is apparent that translational repression via this process leads to reprogramming of gene expression by selectively translating a subset of mRNAs. For example, ATF4 can be translationally upregulated during eIF2alpha phosphorylation to encode for transcription factors that are required to activate downstream stress response genes such as CHOP and GADD34. GADD34 plays a crucial role in recovery of overall translation following ER
Figure 2.11  Prevention of Leaky Scanning by Ribosomes at the AUG of Mouse GADD34 5' UTR Upstream uORF (uORF1) by Introduction of a Kozak Consensus Sequence

HepG2 cell lines stably expressing YFP reporter RNAs with mouse GADD34 5' UTR uORF1 fused to the main YFP ORF (bottom left schematic) or with mutation that strengthens the context of mouse GADD34 5' UTR uORF1 AUG by introducing the Kozak consensus sequence (bottom right schematic). Cells were lysed, and the lysates were subjected to SDS-PAGE, and YFP proteins were detected by Western blotting (top). Total YFP and GAPDH RNAs were also detected by Northern blotting (bottom).
stress through PP1-mediated dephosphorylation of eIF2alpha (Novoa et al., 2001; Novoa et al., 2003). A defect in GADD34 expression in cells can lead to premature apoptosis upon ER stress. This is likely due to failure to express stress response genes (Novoa et al., 2001; Novoa et al., 2003). Thus, the increased expression of GADD34 results in a negative feedback loop to enhance the translation of stress-induced mRNAs during eIF2alpha phosphorylation. It has been shown that transcriptional activation of GADD34 via ATF4 and CHOP is important for the ER stress response (Ma and Hendershot, 2003; Marciniak et al., 2004). In this chapter, we showed that GADD34 is also translationally upregulated by associating with heavier polysomes during ER stress (Figure 2.2). In addition, expression of GADD34 protein increased under thapsigargin treatment in the presence of the transcription inhibitor actinomycin D (Figure 2.4). This result indicated that ongoing transcription was not required for GADD34 induction and that existing basal GADD34 mRNAs can be translated during ER stress. We also showed that endogenous GADD34 mRNAs were associated with higher molecular weight polysomes in cells treated with thapsigargin and actinomycin D (Figure 2.5). However, the induction of GADD34 protein was moderate in thapsigargin/actinomycin D-treated cells compared to cells treated with thapsigargin alone, and was not sufficient to reduce eIF2alpha phosphorylation at later time points (Figure 2.4). This suggests that GADD34 requires both transcriptional and translational controls for maximum expression. The tight regulation of GADD34 level is crucial, as it has previously been shown that defects in GADD34 expression are detrimental to cells. For example, impaired expression of
GADD34 in CHOP−/− mice embryonic fibroblasts leads to persistent eIF2alpha phosphorylation and loss of protein synthesis recovery (Marciniak et al., 2004).

2.4.2 Translational Regulation of GADD34 through 5’ UTR uORFs

We also showed that the 5’ UTR of both human and mouse GADD34 mRNAs is essential and sufficient to direct efficient translation during ER stress. Specifically, the reporter RNAs containing the GADD34 5’ UTR associated with heavier polysomes during eIF2alpha phosphorylation, which is similar to the distribution of endogenous GADD34 mRNAs (Figure 2.7). Moreover, our data indicate that the uORFs of the human and mouse GADD34 5’ UTR play a significant role under basal and stress conditions. Without these uORFs, the reporter RNAs were highly translated basally, but repressed during ER stress (Figures 2.7B and Figure 2.8). This observation is in agreement with the idea that uORFs pose as barriers to the scanning ribosomes, preventing them from reaching the main AUG (Sachs and Geballe, 2006). Further work revealed that the uORF2 of GADD34 is recognized by the scanning ribosomes, and required for the translational control of the transcript, whereas the ribosomes bypass uORF1, which appears to be dispensable in this regulation (Figures 2.8 and 2.10). The scanning ribosomes can skip AUG initiation codons if they do not contain Kozak consensus sequence for optimal initiation (Kozak, 1991). Nucleotide sequence analysis revealed that the mouse GADD34 uORF1 AUG was missing Kozak consensus nucleotides at both -3 and +4, while uORF2 AUG only lacks the consensus nucleotide at +4, strongly suggesting that the lack of the consensus nucleotides at the uORF1 AUG causes leaky
scanning. Indeed, strengthening the context of uORF1 AUG by site directed mutagenesis resulted in most ribosomes now initiating at the main AUG (Figure 2.1).

5’ UTR uORFs have been found to be ubiquitous in eukaryotes, and important in regulating translation of mRNAs under different cellular conditions (Calvo et al., 2009; Iacono et al., 2005; Sachs and Geballe, 2006). Select 5’ UTR uORFs control translation of downstream ORFs by allowing the ribosomes to reinitiate at their downstream ORF, and this mechanism has been most extensively studied in ATF4 and yeast GCN4 (Hinnebusch, 2005; Lu et al., 2004). Normally, the scanning ribosomes recognize and translate the upstream uORF, and reinitiate at the downstream uORF before reaching the main ORF AUG. After eIF2alpha phosphorylation, however, the TC becomes scarce, and following translation of the upstream uORF, there is a higher chance of the scanning ribosomes to acquire the TC past the downstream uORF AUG, thereby reinitiating at the main AUG. In the case of ATF4 and GCN4, translation of the upstream uORF, which is known to be stimulatory for the resumption of the scanning, occurs readily. This is followed by translation of the downstream uORF, which is inhibitory for reinitiation, causing the ribosomes to dissociate from the transcript. Efficiency of reinitiation is known to be dictated by several factors. First, the longer the upstream uORF is, the less efficient reinitiation at the downstream ORF becomes, as it has previously been postulated that the ribosomes are more prone to lose initiation factors required for subsequent reinitiation while translating a long uORF (Kozak, 2001; Poyry et al., 2004). In addition, the length of intercistronic space between the uORFs is also crucial, since if this region is too short, the ribosomes, following translation of the upstream uORF, are more likely to bypass the next uORF before obtaining initiation factors required for
reinitiation (Kozak, 1987b). 5’ UTRs of yeast GCN4 and human ATF4 all satisfy the requirements for efficient reinitiation by having a very short upstream uORF (3 amino acids long) and relatively long intercistronic space separating the uORFs. Based on the data in the current chapter, however, GADD34 uORFs appear to regulate translation via a mechanism distinct from the conventional reinitiation mode. Unlike the upstream uORF in yeast GCN4 and ATF4, the upstream uORF of GADD34 5’ UTR was found to be poorly translated via leaky scanning of the ribosomes. Without the upstream uORF, the downstream uORF of GADD34 5’ UTR was capable of inhibiting translation of the main ORF basally, yet activating it under ER stress. The inhibitory downstream uORF in ATF4 could also regulate translation under eIF2alpha phosphorylation alone, but the regulation was not as great as the WT (Lu et al., 2004), in contrast to GADD34 where the downstream uORF-mediated regulation was comparable to the WT. Furthermore, the uORFs in GADD34 are much larger and the intercistronic space between them is considerably shorter than the ones found in reinitiation, which all argue against a possibility of conventional reinitiation in the translational regulation of GADD34. Lastly, a presence of overlap between uORFs in GADD34 of select organisms such as hamster and mouse makes reinitiation even more unlikely due to an extremely rare back-translocation of the ribosomes (Kozak, 2001).

In summary, GADD34 was found to be translationally regulated during ER stress, in addition to its previously known transcriptional activation. This translational regulation was mediated through the 5’ UTR of GADD34, and more specifically, its downstream uORF which precedes the main ORF. The upstream uORF in its 5’ UTR is skipped by majority of the scanning ribosomes because of its lack of Kozak consensus sequence, and
found to be dispensable for *GADD34* translational regulation basally or under eIF2alpha phosphorylation. On the other hand, the downstream uORF was sufficient to provide translational resistance during ER stress, and this regulation appears to be through a unique mechanism which is distinct from well known reinitiation. Overall, these results suggest that much complexity exists in the field of regulation of gene expression through uORFs.
CHAPTER III

Characterization of the GADD34 5’ UTR uORF2

3.1 Introduction

3.1.1 Conservation of the GADD34 5’ UTR

As discussed in the previous chapter, the GADD34 5’ UTR uORF2 is necessary to mediate translational regulation during eIF2alpha phosphorylation. Under basal conditions, the 5’ UTR uORF2 of human and mouse GADD34 inhibits scanning ribosomes from reaching the main ORF. However, during ER stress, ribosomes can reach the main ORF and initiate translation. This chapter focuses on the role of the GADD34 uORF2 and the nucleotides surrounding uORF2 in translational regulation during basal and ER-stressed conditions.

Due to its key role in translational regulation, the 5’ UTR of GADD34 was further analysed for conservation at the amino acid and nucleotide sequence level. While the amino acid sequence of the GADD34 uORF1 from mammalian species varies, uORF2 is well conserved across select mammalian species (Figure 3.1A and B). All GADD34 uORF2s are 26 codons long, and have ~60-70% identity and similarity in its amino acid sequence (Figure 3.1B).

Several uORFs have been shown to regulate translation based on the amino acid identity of the translated uORFs. The S-Adenosylmethionine decarboxylase (AdoMetDC) mRNA encodes an essential enzyme in polyamine biosynthesis (Hanfrey et al., 2002).
Amino Acid Sequence Conservation within the 5' UTR of \textit{GADD34}.

Figure 3.1  Amino Acid Sequence Conservation within the 5' UTR of \textit{GADD34}

Amino acid sequence alignment of \textit{GADD34} uORF1 (A) and uORF2 (B) from select mammalian species including human (NM_014330), mouse (NM_008654), hamster (L28147), rat (NM_133546), and chimpanzee (XM_001171873, all from GenBank). Identical residues are coloured in red, while residues with chemically similar side chains are coloured blue. For \textit{GADD34} uORF2, amino acid similarity and identity are calculated, and shown below the alignment.

\begin{tabular}{|l|l|}
\hline
\textbf{Human} GADD34 & MQDAARPRARLSPRHLRQPEIL \\
\textbf{Mouse} GADD34 & MRYPARPRIPLPAGTAFAKTACETLRPRAPLRAT \\
\textbf{Hamster} GADD34 & MRHPARPPHPFGTLFAAVCESTRPRAPLRAT \\
\textbf{Rat} GADD34 & MRDPARLRIPLPQPLQFVRHVPPEHA \\
\textbf{Chimp} GADD34 & MQDAARPRARLSPLHLRQPEIL \\
\hline
\end{tabular}

\begin{tabular}{|l|l|}
\hline
\textbf{Human} GADD34 & MNALASLTVRTCDRFWQTEPALPPG \\
\textbf{Mouse} GADD34 & MNPLASRAVRTHDRFWQPEALQPPG \\
\textbf{Hamster} GADD34 & MNALALRDLRTRDFWHPHEPALQPPG \\
\textbf{Rat} GADD34 & MNALAIRLTRDRFWQTEPDLQPPG \\
\textbf{Chimp} GADD34 & MNALASLTVRTCDRFWQTEPALPPG \\
\hline
\end{tabular}

\begin{itemize}
\item \(\rightarrow\) 16 out of 26 residues (62\%) identical throughout the species
\item \(\rightarrow\) 18 out of 26 residues (69\%) similar throughout the species
\end{itemize}
and its 5’ UTR contains two overlapping uORFs, of which the downstream uORF encodes a highly conserved polypeptide. This polypeptide is required for repressing translation of the AdoMetDC ORF during high levels of polyamines (Hanfrey et al., 2005). Another example is shown in yeast CPA1 that encodes a small subunit of arginine-specific carbamoyl phosphate synthetase (Davis, 1986). Its 5’ UTR contains a single uORF which encodes the arginine attenuator peptide (AAP). The AAP contains key conserved amino acid residues crucial for arginine-dependent ribosome stalling at its termination codon, thus preventing scanning ribosomes from reaching the downstream AUG of CPA1 (Hood et al., 2007; Wang et al., 1999). Genome-wide analysis has shown that the conserved peptide uORFs are commonly associated with downstream regulatory genes (Hayden and Jorgensen, 2007).

In addition to the amino acid sequence, 68% of the primary sequence within GADD34 uORF2 is conserved (Figure 3.2A). Moreover, the intercistronic space between the stop codon of GADD34 uORF2 and the initiation codon of the main ORF also contains a significant degree of conservation in its nucleotide sequence (62~73%) (Figure 3.2B). Conservation in nucleotide sequence could suggest existence of a secondary structure important for GADD34 translational control. One possibility is that an internal ribosome entry site (IRES) may direct translation of GADD34. IRESs are generally highly structured RNA elements commonly found in the 5’ UTR of some viral and cellular mRNAs, and can directly recruit ribosomes with a limited set of initiation factors (Hellen and Sarnow, 2001). For example, the 5’ UTR of CAT-1 mRNA contains an uORF that can direct the formation of an IRES during amino acid starvation (Fernandez et al., 2002). Together, the conservation of amino acid and nucleotide sequence found
Figure 3.2  Nucleotide Sequence Conservation within the 5' UTR of GADD34

Nucleotide sequence alignment of GADD34 uORF2 (A) and the intercistronic space between uORF2 and the main ORF (B) from select mammalian species including human (NM_014330), mouse (NM_008654), hamster (L28147), rat (NM_133546), and chimpanzee (XM_001171873, all from GenBank™). For GADD34 uORF2, nucleotide identity is calculated, and shown below the alignment. For the intercistronic space, conserved nucleotides are coloured in red.
within *GADD34* 5’ UTR may play a role in translational regulation during ER stress. This chapter examines whether the conservation at the amino acid and nucleotide levels is important for *GADD34* uORF-mediated translation during ER stress when eIF2alpha is phosphorylated.

### 3.1.2 Activation of Transcriptional Factor Nuclear Factor (NF)-Kappa Beta by eIF2alpha Phosphorylation

In addition to inhibition of overall translation, eIF2alpha phosphorylation also leads to induction of NF-Kappa Beta activity. NF-Kappa Beta is a nuclear transcription factor family that is activated in response to a variety of stimuli such as stress, pathogens, viral infections, and cytokines (Li and Verma, 2002). NF-Kappa Beta functions to activate transcription of numerous target genes, including antimicrobial peptides, stress response genes, and apoptotic proteins. NF-Kappa-Beta plays a key role in the control of innate and adaptive immunity (Pahl, 1999; Verma, 2004). Basally, the inhibitor of Kappa Beta (IKappa Beta) binds to and prevents the nuclear localization of NF-Kappa Beta (Karin and Ben-Neriah, 2000). Upon stimulation, IKappa-Beta becomes phosphorylated at conserved serine residues (Ser-32 and Ser-36) by the IKappa-Beta kinase (IKK) complex, and is subsequently ubiquitinated and targeted for degradation. NF-kappa beta is then released and enters the nucleus to activate transcription of its target genes (Tergaonkar, 2006; Verma, 2004).

eIF2alpha phosphorylation leads to activation of NF-Kappa Beta. Currently, there are two conflicting reports of how this occurs. In one study, phosphorylation of
eIF2alpha by PERK and GCN2 resulted in the release, but not degradation, of IKappa-Beta from NF-Kappa Beta, resulting in its activation (Jiang et al., 2003). In the second study, eIF2alpha phosphorylation by PERK promoted phosphorylation of and subsequent degradation of IKappa-Beta, thus leading to NF-Kappa Beta activation (Deng et al., 2004).

NF-Kappa Beta is also known to be exploited by viruses, such as human immunodeficiency virus type 1 (HIV-1) and CMV, which contain NF-Kappa Beta-dependent promoters to activate transcription of their viral genes (Hiscott et al., 2001; Karin and Ben-Neriah, 2000). Hence, it should be noted that transcription of all of the reporter constructs is under the CMV promoter. As such, a major consideration in my studies has taken into account the transcriptional responses of the reporter plasmids during ER stress. The goal of this chapter is to explore the GADD34 translational activation via its 5’ UTR under ER stress in more detail, specifically via the uORF2.

3.2 Materials and Methods

3.2.1 Construction of GADD34 5’ UTR Mutants

To construct YFP pcDNA3 mammalian expression plasmids containing the GADD34 5’ UTRs, we first cloned the 5’ UTRs into pGEM3 which contains YFP. Subsequently, the GADD34 5’ UTR-YFP was subcloned into pcDNA3. Briefly, the uORF2 of mouse GADD34 5’ UTR was PCR-amplified from a WT mouse GADD34 5’ UTR-YFP reporter construct as a template by using primers, PrEJ666 and PrEJ667,
which contain a 5’ EcoRI and a 3’ NcoI site. The PCR fragment was cloned into EcoRI and NcoI sites of the pGEM3 vector containing an YFP ORF. Next, the GADD34 uORF2-YFP ORF fragment was subcloned into EcoRI and XbaI sites of the pcDNA3 vector to create pEJ341 (mGADD34 2nd uORF-YFP WT) (Invitrogen). The frameshift mutant (mGADD34 2nd uORF-YFP Frameshift) (pEJ343) was created by using the QuikChange site-directed mutagenesis kit (Stratagene) to add a nucleotide in the 3rd codon of mouse GADD34 uORF2 using primers PrEJ672F/R (Table 3.1). This shifted the reading frame of the uORF2 in the +1 frame. To put it back into frame, a nucleotide within the 25th codon was removed, thus restoring the original reading frame using PrEJ674F/R (Table 3.1). The truncated uORF2 mutant (mGADD34 2nd uORF-YFP 26aa to 15aa) (pEJ342) was made by introducing a premature stop codon within the mouse GADD34 uORF2 at the 16th codon position by QuikChange with 26to15aa F/R primers. The Mouse GADD34 uORF2 mutant containing an optimal Kozak consensus sequence (GAC ATG A → GAC ATG G) (mGADD34 uORF2-YFP Strengthened) (pEJ345) was also created by QuikChange using uORF2St F/R primers. The intercistronic space between the uORF2 and the main YFP ORF was increased by using a two-way PCR strategy in which one set of primers, DBLIS1 F/R, was used to amplify the uORF2 with a 3’ overhang containing a series of additional nucleotides. The other set of primers, DBLIS2 F/R, amplified the YFP ORF with a 5’ overhang with the nucleotides complementary to the 3’ overhang of the DBLIS1 F/R. The resulting PCR fragment was ligated into EcoRI and XbaI sites of pcDNA3 to create pEJ344 (mGADD34 2nd uORF-YFP IS Double). The uORF2 of the mGADD34 2nd uORF-YFP WT reporter construct was swapped with the first 26 codons of human GAPDH (AF261085.1 in GenBank™) by
PCR with two overlapping primers, GAPDH26 F/R. This PCR product was cloned into EcoRI and NcoI sites of pcDNA3 vector containing YFP ORF to create pEJ347 (mGADD34 2\textsuperscript{nd} uORF-YFP 26aa GAPDH). Lastly, the mutant with all cytosines changed to guanines in the intercistronic space between the uORF2 and the main ORF (mGADD34 2\textsuperscript{nd} uORF-YFP IS C to G) (pEJ348) was created in the similar manner using primers, ISCtoG F/R (see Table 3.1)

3.2.2 Translational Reporter Assays in HepG2 Cells

To monitor GADD34 regulation by its 5’ UTR, we followed a strict transient transfection protocol. On Day 1, 9.6 µg of DNA was transfected (Lipofectamine 2000, Invitrogen) into HepG2 cells that were plated in a 10 cm tissue culture plate (40\% confluency). On Day 2, after confirming the transfection efficiency (at least greater than 60\%) by detection of YFP expression, the cells were split into a 6-well tissue culture plate. By performing a single transfection and then splitting the cells, we ensured that each reporter would have similar transfection efficiencies, so that it can be compared within each experiment. On Day 3, the transfected HepG2 cells, now at approximately 80-100 \% confluency, were incubated in fresh DMEM at 37 °C for 1.5 hr before they were treated with 1 µM thapsigargin in the presence or absence of 5 µg/ml of actinomycin D for 6 hr. Actinomycin D was added 10 min prior to thapsigargin addition. Following 6 hr incubation, the cells were harvested, and RNA or protein lysates were extracted. For protein extraction, the cells were washed twice with 1 X PBS with 10 mM NaF and 17.5 mM β-glycerophosphate, and scraped into lysis buffer containing 20 mM
<table>
<thead>
<tr>
<th>Primer #</th>
<th>Sequence</th>
<th>Desired Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrEJ666</td>
<td>5' - ACTAGAATTCGACATGAAACCCTGTGCTTCGCGAG3'</td>
<td>mGADD34 2nd uORF-YFP WT (pEJ341)</td>
</tr>
<tr>
<td>PrEJ667</td>
<td>5' - GTACCATGGGCTGTTGGCGCGGCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP WT (pEJ341)</td>
</tr>
<tr>
<td>PrEJ672F</td>
<td>5' - GCTAGAGCATGAAACCCTGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP Frameshift (Add 1) (pEJ343)</td>
</tr>
<tr>
<td>PrEJ672R</td>
<td>5' - GAGGTCGAGCGCTGGCACTGTAACCCCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP Frameshift (Add 1) (pEJ343)</td>
</tr>
<tr>
<td>PrEJ674F</td>
<td>5' - GCGCGCTCAACCTGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP Frameshift (Remove 1) (pEJ343)</td>
</tr>
<tr>
<td>PrEJ674R</td>
<td>5' - GGCGGCGGGCTGCACGTGCACTGACCCCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP Frameshift (Remove 1) (pEJ343)</td>
</tr>
<tr>
<td>26to15aa F</td>
<td>5' - GACCGACGTAATTCGAGCGCGGCGGCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP 26aa to 15aa (pEJ342)</td>
</tr>
<tr>
<td>26to15aa R</td>
<td>5' - AAGCCTGCGGTTGTTGTTGCAAAACGCAGCGCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP 26aa to 15aa (pEJ342)</td>
</tr>
<tr>
<td>uORF2St F</td>
<td>5' - GTCTGACGCTGCTGCACCTGACCCCGGGCTGC-3'</td>
<td>mGADD34 uORF2-YFP Strengthened (pEJ345)</td>
</tr>
<tr>
<td>uORF2St R</td>
<td>5' - CTCGCGAGCCAGCGGCTGCTACAGCGGACGCAGCGCGGGCTGC-3'</td>
<td>mGADD34 uORF2-YFP Strengthened (pEJ345)</td>
</tr>
<tr>
<td>DBLIS1 F</td>
<td>5' - CTAGAATTCGACATGAAACCCTGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP IS Double (amplify uORF2-IS) (pEJ344)</td>
</tr>
<tr>
<td>DBLIS1 R</td>
<td>5' - ATCGTACGTTGAAAGCTGACCTGACCCCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP IS Double (amplify uORF2-IS) (pEJ344)</td>
</tr>
<tr>
<td>DBLIS2 F</td>
<td>5' - GTCTGAGTTGAGTCACGGTACCCCGGGCTGC-3'</td>
<td>mGADD34 2nd uORF-YFP IS Double (amplify YFP-IS) (pEJ344)</td>
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<tr>
<td>DBLIS2 R</td>
<td>5' - GTATCTAGAGTCGAGCGCTGCCTTACTC-3'</td>
<td>mGADD34 2nd uORF-YFP IS Double (amplify YFP-IS) (pEJ344)</td>
</tr>
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<td>GAPDH26 F</td>
<td>5' - CGAAGATTCGACATGAAACCCTGCTGC-3'</td>
<td>mGADD34 uORF2-YFP 26aa GAPDH (pEJ347)</td>
</tr>
<tr>
<td>GAPDH26 R</td>
<td>5' - GACCCATGCTGAGTTGGCGCGGCGGCTGCACCGCAGCCAGCGGGCTGC-3'</td>
<td>mGADD34 uORF2-YFP 26aa GAPDH (pEJ347)</td>
</tr>
<tr>
<td>ISCtoG F</td>
<td>5' - CAGAATTCGACATGAAACCCTGCTGC-3'</td>
<td>mGADD34 uORF2-YFP IS C to G (pEJ348)</td>
</tr>
<tr>
<td>ISCtoG R</td>
<td>5' - AGCGCATGCTGCCCTCCCTCCTACACCGCGGCGGGCTGC-3'</td>
<td>mGADD34 uORF2-YFP IS C to G (pEJ348)</td>
</tr>
</tbody>
</table>

Table 3.1  List of Primers Used to Construct Selected GADD34 5' UTR Mutants

The name and sequence of primers used to create various GADD34 5' UTR mutants by PCR are given.
HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100 (v/v), 10% glycerol, 1 mM EDTA, 10 mM NaF, 17.5 mM β-glycerophosphate, and a protease inhibitor mixture (Roche Applied Science). The lysates were then prepared for SDS-PAGE and Western blotting in the same way as described in Chapter 2. For RNA extraction, the cells were lysed with Trizol reagent (Invitrogen), followed by RNA precipitation with isopropanol. Equal amounts of purified RNAs were then loaded on 1% denaturing RNA gel (1 X MOPS and 20% Formaldehyde), and transferred to a Zeta-probe membrane (Bio-Rad) for Northern blotting.

3.3 Results

3.3.1 Polysome Association of Mutant GADD34 Reporter mRNAs during ER Stress

Based on sequence conservation within the uORF2 and the intercistronic space, we hypothesize that these regions are sufficient for GADD34 translational regulation. Since the previous GADD34-YFP reporter RNAs contains the full-length 5’ UTR, we asked whether the uORF2 and the intercistronic space are sufficient for regulation during ER stress by using reporter YFP RNAs containing only the uORF2 and intercistronic space (mGADD34 2nd uORF-YFP WT). Cells stably expressing this reporter RNA were treated with or without 1 µM thapsigargin for 30 minutes, and then subjected to polysome analysis (Figure 3.3A). As seen previously, endogenous GADPH RNAs were mostly found in high molecular weight fractions #8 to 10 in untreated cells. However, ER stress,
Figure 3.3  Polysomal Association of the YFP Reporter mRNA Containing the Mouse GADD34 uORF2 and Intercistronic Space

(A) Northern blots (see Materials and Methods 2.2.6) of the YFP reporter and GAPDH mRNAs collected from polysomal analysis on lysates of HepG2 cells stably expressing the mutant reporter construct (shown above) after 1 μM thapsigargin treatment (thap) or mock-treatment (unt) for 30 minutes. (B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage of total radioactivity in all fractions within a single sucrose gradient.
the mRNAs shifted to lower molecular weight polysomal fractions, indicating that this mRNA is translationally repressed (Figure 3.3). Interestingly, the mGADD34 2\textsuperscript{nd} uORF-YFP WT reporter RNA exhibited a shift to heavier molecular weight polysomal fractions during thapsigargin treatment, similar to that of the reporter RNA containing the WT 5’ UTR of GADD34 (Figure 2.7C). This indicated that the uORF2 and the intercistronic space of the GADD34 5’ UTR are sufficient to mediate its basal repression and activation of GADD34 expression under ER stress. Moreover, this result supports our earlier conclusion that the uORF1 of GADD34 is dispensable for this regulation.

We next asked whether the conserved amino acid sequence within uORF2 of GADD34 is important for regulation. To change the amino acid sequence of the uORF2, the reading frame of mouse GADD34 uORF2 was shifted to the +1 frame at the 3\textsuperscript{rd} codon by inserting an extra nucleotide and restored to the original reading frame by removing a nucleotide at the 25\textsuperscript{th} codon. This frameshift mutation altered amino acids 3-24 of the uORF2 (Figure 3.4A), but kept the nucleotide sequence and its base composition intact. We also ensured that no premature stop codons were introduced within the uORF2 by these mutations (Figure 3.4A). The mGADD34 2\textsuperscript{nd} uORF-YFP Frameshift reporter RNA (pEJ343) is largely found in fractions #6 and #7, indicating that the reporter RNA is translationally repressed during basal conditions (Figure 3.4). However, the mutant RNA shifted to the heavier molecular weight fractions #7 and #8 upon ER stress, suggesting that this mRNA is translationally active during ER stress (Figure 3.4). This finding indicated that the amino acid identity of the uORF2 is not required for translational regulation of GADD34.
Figure 3.4  Polysomal Association of the YFP Reporter mRNA Containing a Frameshift Mutation in Mouse GADD34 uORF2

(A) Northern blots of the YFP reporter and GAPDH mRNAs collected from polysomal analysis on lysates of HepG2 cells stably expressing the mutant reporter construct (shown above) after 1 μM thapsigargin treatment (thap) or mock-treatment (unt) for 30 minutes.

(B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage out of total radioactivity in all fractions within a single sucrose gradient.
Because the amino acid sequence did not alter the regulation of translation under basal and ER-stress conditions, we asked whether the size of the uORF2 plays a role in regulating translation. All of the mammalian \textit{GADD34} uORF2 are 26 codons long (Figure 3.1B). To test if the conserved size of the uORF2 is important, a premature codon was introduced at the 16\textsuperscript{th} codon position of the uORF2 by site directed mutagenesis, which produces a truncated 15 amino acid-long peptide (Figure 3.5A). Basally, the \textit{mGADD34} \textsuperscript{2}\textsuperscript{nd} uORF-\textit{YFP} 26aa to 15aa reporter RNAs (pEJ342) sedimented to heavy molecular weight fractions (mostly in fraction #8) in untreated cells (Figure 3.5). In contrast, the \textit{mGADD34}-\textit{YFP} or the \textit{mGADD34} \textsuperscript{2}\textsuperscript{nd} uORF-\textit{YFP} reporter RNAs containing the wild-type 5' UTR or only the uORF2 sedimented to fractions #6 and #7, as shown in Figures 2.7 and 3.3. Thus, this result suggested that shortening the uORF2 does not keep translation of the ORF repressed under basal condition. Upon thapsigargin treatment, the distribution of the \textit{mGADD34} \textsuperscript{2}\textsuperscript{nd} uORF-\textit{YFP} 26aa to 15aa reporter RNA was unchanged, indicating that this mRNA was not translationally upregulated during ER stress (Figure 3.5B). This result suggests that the conserved length of the uORF2 is critical for suppressing \textit{GADD34} expression under basal conditions.

Because the \textit{GADD34} uORF2 appears to be the main feature regulating translation, we wanted to re-examine whether the uORF2 is translated. Previous work indicated that uORF2 is recognized by scanning ribosomes efficiently. However, a closer inspection of the \textit{GADD34} uORF2 AUG initiation codon showed that the AUG is in sub-optimal Kozak consensus. The Kozak consensus sequence for ribosome recognition is a purine residue (adenine or guanine) at the -3 position and a guanine at the +4 position, given that the A of the AUG is +1. (Kozak, 1984). The \textit{GADD34} uORF2 AUG contains a
Figure 3.5  Polysomal Association of the YFP Reporter mRNA Containing a Truncation Mutation in Mouse GADD34 uORF2

(A) Northern blots of the YFP reporter and GAPDH mRNAs collected from polysomal analysis on lysates of HepG2 cells stably expressing the mutant reporter construct (shown above) after 1 μM thapsigargin treatment (thap) or mock-treatment (unt) for 30 minutes.

(B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage out of total radioactivity in all fractions within a single sucrose gradient.
guanine residue at the -3 position and an adenine residue at the +4, suggesting that the AUG codon is suboptimal (Figure 3.6A). To test whether the context of the AUG codon is important for regulation, the +4 adenine residue in mouse GADD34 uORF2 AUG was mutated to a guanine residue, thus “strengthening” its context (Figure 3.6A). When the cells expressing the mutant mGADD34 2nd uORF-YFP Strengthened reporter RNAs (pEJ345) were subjected to polysomal analysis under basal conditions, the majority of the mutant reporter RNA sedimented to fraction #7, thus associating with few ribosomes (Figure 3.6). Under ER stress, the mutant reporter RNA associated with more translating ribosomes, as most of the mRNA sedimented to fractions #7 to #9 (Figure 3.6). Overall, strengthening of the context of the uORF2 AUG minimally affected translational regulation of GADD34 under eIF2alpha phosphorylation.

The spacing between the uORF2 and the downstream main ORF shows a significant degree of conservation in its nucleotide sequence (Figure 3.2B). The intercistronic space is particularly GC-rich, aligns well among mammalian species, and ranges from 22-26 nucleotides long (Figure 3.2B). To test whether the intercistronic distance is important, we created a mutant YFP reporter construct in which the distance between the mouse GADD34 uORF2 and YFP ORF is extended from 23 to 46 nucleotides by duplicating the 23 nucleotide spacer (mGADD34 2nd uORF-YFP IS Double) (ppEJ344) (Figure 3.7A). The polysomal distribution of this mutant reporter RNA exhibited a similar pattern as the WT GADD34 5’ UTR. The mutant RNA mostly sedimented to fraction #7 in untreated cells, and shifted to heavier molecular weight fractions during thapsigargin treatment (Figure 3.7). The result indicated that doubling the intercistronic distance has
Figure 3.6 Polysomal Association of the YFP Reporter mRNA Containing a Strengthening Mutation in Mouse GADD34 uORF2

(A) Northern blots of the YFP reporter and GAPDH mRNAs collected from polysomal analysis on lysates of HepG2 cells stably expressing the mutant reporter construct (shown above) after 1 μM thapsigargin treatment (thap) or mock-treatment (unt) for 30 minutes.

(B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage out of total radioactivity in all fractions within a single sucrose gradient.
Figure 3.7  Polysomal Association of the YFP Reporter mRNA Containing a Doubled Intercistronic Space in Mouse GADD34 5' UTR

(A) Northern blots of the YFP reporter and GAPDH mRNAs collected from polysomal analysis on lysates of HepG2 cells stably expressing the mutant reporter construct (shown above) after 1 μM thapsigargin treatment (thap) or mock-treatment (unt) for 30 minutes.

(B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage out of total radioactivity in all fractions within a single sucrose gradient.
little influence on polysomal association and translational regulation of \textit{GADD34} via its 5’ UTR during ER stress.

We next asked whether the GC content of the intercistronic space is important. To determine if the intercistronic space in \textit{GADD34} contains any conserved secondary structures, M-Fold online software was used to predict possible folding of RNA within the intercistronic space (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) (Figure 3.8). In this region, a single stemloop structure was predicted via cytosines-guanines base pairing (Figure 3.8). Therefore, we mutated cytosines (pyrimidines), the most abundant nucleotides in the region, to guanines (purines) (Figure 3.9A). I reasoned that this would remove any C-G base pairings within the intercistronic space, which should disrupt any possible RNA secondary structures. In untreated cells, the m\textit{GADD34} 2\textsuperscript{nd} uORF-\textit{YFP IS C to G} (pEJ348) reporter RNA was found mostly in fractions #6 through #8. During thapsigargin treatment, the majority of the reporter RNA sedimented mainly to fraction #8 (Figure 3.9). Interestingly, most of the reporter RNA with C to G mutations did not sediment in a single fraction like the other reporter RNAs, but sedimented throughout the multiple fractions (fractions #6-8) under basal condition (Figure 3.9). However, ER stress by thapsigargin treatment resulted in the reporter RNAs mostly found in fraction #8, suggesting that this mRNA is shifting to higher molecular weight fractions (Figure 3.9). Thus, changing the cytosines to guanines in the intercistronic space of \textit{GADD34} does not affect the overall translational regulation during ER stress.

3.3.2 Effects of Mutations in 5’ UTR of \textit{GADD34} on Proteins Synthesis \textit{in vivo}

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Figure 3.8 Predicted Secondary Structures of the *GADD34* 5' UTR Intercistronic Space by M-Fold

Predicted Secondary Structures of *GADD34* 5' UTR intercistronic space from (A) human (NM_014330), (B) mouse (NM_008654), (C) hamster (L28147), (D) rat (NM_133546), and (E) chimpanzee (XM_001171873, all from GenBank™) by M-Fold (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). The optimal free energy values of each predicted structure is indicated. When there are multiple possible secondary structures, one with the lowest free energy (most stable) is shown.
Figure 3.9  Polysomal Association of the YFP Reporter mRNA Containing Cytosine to Guanine Mutations in the Intercistronic Space of Mouse GADD34 5' UTR

(A) Northern blots of the YFP reporter and GAPDH mRNAs collected from polysomal analysis on lysates of HepG2 cells stably expressing the mutant reporter construct (shown above) after 1 μM thapsigargin treatment (thap) or mock-treatment (unt) for 30 minutes. (B) Quantification of the Northern blots from (A). The amount of a radioactive probe is measured as a percentage out of total radioactivity in all fractions within a single sucrose gradient. Note that fraction #1 in both Unt and Thap samples was lost, and its radioactivity is arbitrarily given as 0%.
We next determined the effects of the various mutations in \emph{GADD34} 5’ UTR on protein synthesis under ER stress \textit{in vivo} to verify that these reporter mRNAs are translated. Towards this, we tested the mutant reporters by transient transfection followed by treatment with thapsigargin. Since these reporter RNAs are under the control of the CMV promoter and the CMV promoter can be induced during eIF2alpha phosphorylation, we needed to uncouple the effects of transcription and translation of these reporter constructs (Hiscott et al., 2001; Jiang et al., 2003). Therefore, we also added actinomycin D to inhibit transcription of the reporter constructs to study translational activity of the existing reporter mRNAs in the cell. To determine whether this is a viable approach, we monitored the translation of the m\emph{GADD34}-\emph{YFP} (pEJ339) reporter RNAs under these conditions. When treated with thapsigargin for 6 hr, a significant increase (approximately by 14 fold) in YFP protein level was observed in the m\emph{GADD34}-\emph{YFP} expressing cells as compared to that in untreated cells (Figure 3.10A). By Northern blot analysis, the reporter RNA level increased (approximately by 16 fold), indicating, as expected, that ER stress increases the transcription of this CMV-driven reporter (Figure 3.10A). When the cells were treated with both actinomycin D and thapsigargin, the m\emph{GADD34}-\emph{YFP} (pEJ339) reporter RNA levels were not induced, indicating that transcription was shut off during ER stress (1.1 fold increase) (Figure 3.10A). In contrast, the YFP protein expression increased 3 fold, demonstrating that this reporter mRNA is translationally upregulated by the \emph{GADD34} 5’ UTR during ER stress (Figure 3.10A). When the mutant 1&2 KO m\emph{GADD34}-\emph{YFP} (pEJ340) which contains mutations that knocked out both uORF AUG codons was tested, the basal expression of YFP protein was much higher than that of the pEJ339, showing that the presence of the uORFs is required to repress
Figure 3.10  Translational Upregulation by Mouse GADD34 5' UTR uORFs during ER Stress

Northern blots of YFP and GADPH RNAs (upper panels) and Western blots of YFP protein (lower panels) from HepG2 cells stably expressing YFP reporter constructs with WT (A) and Mutant 1&2 KO mouse GADD34 5' UTR (B). The cells were left untreated (Mock), treated with 1 µM thapsigargin alone (Thaps), or with 1 µM thapsigargin and 5 µg/ml actinomycin D (Thaps + ActD) for 6 hr before they were lysed for RNA and protein extraction. The YFP protein expression was measured by LI-COR Odyssey imaging system (Lincoln, NE), and the value of fold change in each sample was normalized by that of the mock-treated sample. The results shown are representative of an experiment that was repeated at least two times.
translation basally (Figure 3.10A and B). This finding correlates with the previous polysomal analysis in which the same mutant reporter was associated with more ribosomes than the WT in unstressed cells (Figure 2.7B). During thapsigargin treatment, YFP protein expression of the Mutant 1&2 KO m\textit{GADD34-YFP} reporter RNA did not change significantly (1.2 fold), while its RNA levels increased 3.7 fold. In the presence of thapsigargin and actinomycin D, YFP protein levels stayed the same as the basal level (1.0 fold), and the RNA levels also did not change significantly (0.7 fold) (Figure 3.10B). Since YFP has a relatively long half-life (26 hours), the high expression of YFP in the Mutant 1&2 KO m\textit{GADD34-YFP} reporter cell line is due to the steady-state levels of YFP. Because we do not observe an increase in YFP levels during thapsigargin treatment alone or thapsigargin/actinomycin D together, this result shows that this mutant mRNA is translationally repressed. In contrast, the increase in YFP levels in the m\textit{GADD34-YFP} cells under thapsigargin treatment must be due to translational upregulation of this mRNA. In summary, we have set up a relatively quick and efficient method to analyze the translation of a series of mutant \textit{GADD34} reporter constructs.

Using this method, \textit{YFP} reporter constructs containing various mutations within \textit{GADD34} 5' UTR were then tested for their translational regulation under ER stress. All reporter constructs were transiently transfected in HepG2 cells, followed by treatment with thapsigargin alone or thapsigargin/actinomycin D treatment the day after. Cells were harvested after 6 hours, and the protein and RNA levels were analyzed by Western and Northern blot analysis.

First, expression of the m\textit{GADD34} 2\textsuperscript{nd} uORF-\textit{YFP} (pEJ341) reporter RNA was tested. Treatment of thapsigargin and actinomycin D increased YFP levels by 1.9 fold
whereas the RNA levels remained relatively unaltered (0.9 fold) (Figure 3.11). This indicated that the uORF1 is indeed dispensable for translational regulation, and correlates with the previous polysome data that the uORF2 and the intercistronic space are sufficient to mediate polysomal association just as well as the WT (Figure 3.3). Similarly, the frameshifted GADD34 uORF2 (pEJ343) was able to mediate translational regulation under thapsigargin/actinomycin D treatment (1.8 fold) and its RNA level remained unaltered (0.7 fold). This correlated with its polysomal shift to higher molecular weight fractions during ER stress. (Figures 3.12A and 3.4). On the other hand, the mGADD34 2\textsuperscript{nd} uORF-YFP 26aa to 15aa reporter (pEJ342) expressed much higher YFP under basal conditions and slightly induced (1.7 fold) under thapsigargin treatment despite transcriptional activation (8.0 fold) (Figure 3.12B). Moreover, addition of actinomycin D with thapsigargin led to an even smaller increase in the YFP protein level (1.2 fold) although the RNA level was higher (2.7 fold), demonstrating that translational control under ER stress is lost in the absence of the full length GADD34 uORF2 (Figure 3.12B).

To further determine whether the uORF2 nucleotide sequence is important, the uORF2 was completely swapped with the first 26 codons of human GAPDH gene (mGADD34 2\textsuperscript{nd} uORF-YFP 26aa GAPDH) (pEJ347) (Figure 3.13). The first 26 codons of GAPDH were still able to repress the basal translation of the pEJ347 RNA, and increased the YFP expression under thapsigargin and thapsigargin/actinomycin D treatments by 6.8 and 2.3 fold, respectively (Figure 3.13). Transcription activation was only observed under treatment of thapsigargin alone (8.3 fold), but not under treatment of thapsigargin/actinomycin D (0.5 fold) (Figure 3.13). This result suggests that the conservation at the nucleotide or amino acid level within the uORF2 is not necessary for
Figure 3.11  Translational Upregulation by Mouse GADD34 5' UTR uORF2 and the Intercistronic Space during ER Stress

Northern blots of YFP and GAPDH RNAs (upper panels) and Western blots of YFP protein (lower panels) from HepG2 cells stably expressing YFP reporter constructs with mouse GADD34 5' UTR uORF2 and intercistronic space. The cells were left untreated (Mock), treated with 1 µM thapsigargin alone (Thaps), or with 1 µM thapsigargin and 5 µg/ml actinomycin D (Thaps + ActD) for 6 hr before they were lysed for RNA and protein extraction. The YFP protein expression was measured by LI-COR Odyssey imaging system (Lincoln, NE), and the value of fold change in each sample was normalized by that of the mock-treated sample. The results shown are representative of an experiment that was repeated at least two times.
Figure 3.12  Translational Upregulation by Frameshifted uORF2, but Not Truncated uORF2 of mouse GADD34 5' UTR during ER stress

Northern blots of YFP and GADPH RNAs (upper panels) and Western blots of YFP protein (lower panels) from HepG2 cells stably expressing YFP reporter constructs with frameshifted (A) and truncated (B) mouse GADD34 uORF2. The cells were left untreated (Mock), treated with 1 µM thapsigargin alone (Thaps), or with 1 µM thapsigargin and 5 µg/ml actinomycin D (Thaps + ActD) for 6 hr before they were lysed for RNA and protein extraction. The YFP protein expression was measured by LI-COR Odyssey imaging system (Lincoln, NE), and the value of fold change in each sample was normalized by that of the mock-treated sample. The results shown are representative of an experiment that was repeated at least two times.
Figure 3.13  Translational Upregulation by Mouse GADD34 5' UTR uORF2 Containing the First 26 Codons of Human GAPDH during ER Stress

Northern blots of YFP and GAPDH RNAs (upper panels) and Western blots of YFP protein (lower panels) from HepG2 cells stably expressing YFP reporter constructs with mouse GADD34 5' UTR uORF2 and intercistronic space. The cells were left untreated (Mock), treated with 1 µM thapsigargin alone (Thaps), or with 1 µM thapsigargin and 5 µg/ml actinomycin D (Thaps + ActD) for 6 hr before they were lysed for RNA and protein extraction. The YFP protein expression was measured by LI-COR Odyssey imaging system (Lincoln, NE), and the value of fold change in each sample was normalized by that of the mock-treated sample.
GADD34 translational control, as long as a 26 codon-long uORF is present in the 5’ UTR of the transcript.

3.4 Discussion

We have taken two approaches to evaluate translational control by GADD34 5’ UTR elements under ER stress in vivo. The first approach monitored the polysomal association of the reporter RNAs with GADD34 5’ UTR elements. The second approach was by transiently transfecting HepG2 cells with the reporter constructs to detect RNA and protein levels during thapsigargin and actinomycin D treatment.

We show that uORF2 and the intercistronic space is sufficient for translational regulation of GADD34 under ER stress, as the pEJ341 reporter RNA mediated translation just as efficiently as the WT GADD34 5’ UTR during thapsigargin-induced ER stress (Figures 3.3 and 3.11). However, mutating the conserved amino acid sequence did not affect translational regulation of the reporter RNAs, and exhibited the WT-like phenotype during ER stress instead (Figures 3.4 and 3.12A). This suggests that the GADD34 uORF2 regulates translation via a mechanism that is distinct from those in AdoMetDc and CPA1, where their regulation is largely dependent on the peptide sequences of the uORFs (Hanfrey et al., 2005; Wang et al., 1999).

In addition, our data suggests that the conserved size of the GADD34 uORF2 may be important in its translational regulation. Truncating the uORF to 16 amino acids failed to exert basal repression and translational activation under ER stress (Figures 3.5 and 3.12B). It is possible that the shorter uORF2 may allow more reinitiation of ribosomes. It
is known that the efficiency of ribosome reinitiation decreases as an ORF becomes longer (Kozak, 2001; Poyry et al., 2004). Normally, ribosomes that translate short uORFs (2-3 amino acids) reinitiate efficiently whereas longer ORFs tend to prohibit reinitiation (Kozak, 2001). This has been attributed to initiation factors (i.e. eIF4G) that still associate with the 40S even after the ribosome has begun elongating (Poyry et al., 2004).

Compared to the 3 codon long uORF1 present in yeast GCN4 and human ATF4 in which reinitiation of the ribosomes readily occurs, the GADD34 uORF2 is considerably longer (26 codons long), making reinitiation quite unlikely to occur. Shortening the uORF2 to 15 codons may increase the chance of reinitiation to occur, which could explain why there is a dramatic increase in basal translation. Thus, it is likely that the conserved length of the uORF2 may be keeping basal translation of GADD34 low.

During eIF2alpha phosphorylation, the truncated uORF2 did not cause the shift of the pEJ342 reporter RNA to heavier molecular weight fractions like that of the mGADD34-YFP mRNA (Figure 3.5). This suggests that the full length uORF2 is required for translational regulation of GADD34. However, one limitation of polysomal analysis by sucrose density gradient is that it becomes progressively more difficult to resolve RNAs associated with multiple ribosomes towards the bottom (heavier) of the gradient. It is possible that a shift toward heavier molecular weight fractions did occur with the pEJ342 reporter RNA, but we could not resolve this by this method. Thus, polysomal data alone cannot support the requirement of the full length uORF2 for translational control under ER stress. When the pEJ342 reporter RNA was tested for its translation under thapsigargin/actinomycin D, its translational regulation was lost, similar
to that of the pEJ340 (1&2 KO). This indicates that the full length uORF2 is a required feature for translational activation of GADD34 during eIF2alpha phosphorylation.

The “intercistronic space” between the stop codon and the downstream AUG codon within GADD34 5’ UTR is another region that exhibits a high degree of conservation in nucleotide sequence (Figure 3.2B). This region is particularly rich in cytosines and guanines, and in the case of reinitiation in GCN4, the C+G-rich sequences surrounding the inhibitory uORF termination codon were found to prevent reinitiation by promoting dissociation of the ribosomes (Grant and Hinnebusch, 1994). A destabilizing effect of the C+G rich stop codon context on ribosome retention has also been observed in the sequence-independent uORFs of the yeast AP1-like transcription factor YAP2 (Vilela et al., 1999). Therefore, the high C+G content around the GADD34 uORF2 stop codon may function in a similar way to inhibit reinitiation of the ribosomes and thus repress basal translation of the main ORF. In the shortened GADD34 uORF2 mutant (pEJ342), a premature stop codon was introduced after the 15th codon, and its surrounding sequences are no longer C+G rich. It is possible that this could facilitate reinitiation of the ribosomes. This may explain why such high basal translational level is observed with the mGADD34 2nd uORF-YFP 26aa to 15aa (Figures 3.5 and 3.12B).

We also addressed significance of the size of the intercistronic space between the GADD34 uORF2 and the main ORF. Another determinant in efficient reinitiation is the spacing between two adjacent ORFs, as the farther apart they are, the greater chance for the scanning ribosomes to reacquire factors required for subsequent reinitiation (Kozak, 1987b). The mouse GADD34 5’ UTR intercistronic space is 23 nucleotides long which is much smaller than ones in human ATF4 (87 nucleotides) and yeast GCN4 (198
nucleotides between uORF1 and uORF4). Here, we evaluated the effect of the increased intercistronic space length on GADD34 5’ UTR mediated translational regulation. Our polysomal data indicated that increasing the space from 23 to 46 nucleotides did not affect translational regulation of GADD34 (Figure 3.7). It should be noted that the C+G content of the lengthened intercistronic space remained high since its length was doubled by repeating the original sequence side by side, and therefore, the dissociation effect of the C+G rich context may still be present in this case, preventing reinitiation at basal level.

The uORF2 AUG, despite its lack of the strong context, is recognized and translated by the ribosome as well as when its AUG is strengthened (Figures 2.10 and 3.6), indicating that the translation of the uORF2 is already optimized to provide maximum basal repression. When we changed the AUG context to an optimal Kozak context, we also showed that translation regulation under basal and stress condition is not affected. Thus, this suggests that the role of the uORF2 is to prevent ribosomes from reaching the main ORF. This strongly points to the possibility that the uORF2 is indeed being translated, and that reinitiation does not occur under basal conditions. Moreover, this is in support of our previous data that the uORF is translated efficiently both under basal and ER stressed conditions (Lee et al., 2009).

We also explored the importance of nucleotide sequence conservation within the GADD34 uORF2 and its intercistronic space in translational regulation. The high degree of identity at the nucleotide sequence level may highlight possible presence of regulatory structural features in these regions. However, although M-Fold prediction algorithm predicted a single stemloop within the intercistronic space (Figure 3.8), it did not reveal
any conserved structures between the different mammalian *GADD34* for the entire 5' UTRs. Our data suggest that the sequence conservation is not important, since the uORF2 with a completely different nucleotide sequence from *GAPDH* could still regulate translation (Figure 3.13). Furthermore, changing conserved cytosines to guanines in the intercistronic space did not affect the polysomal distribution of the reporter RNA (Figure 3.9). However, this data is still very preliminary, thus warranting further studies on possible significance of cytosines and guanines in this region. The mutated intercistronic region was still rich in guanines, and it is possible that the G-richness might bear some significance in the regulation.

How can the *GADD34* 5' UTR uORF2 mediate its translational regulation under eIF2alpha phosphorylation? By mutational analysis, the current thesis tested various aspects of the uORF2 and the intercistronic space-mediated translational regulation, and I have summarized what we learned from these studies in Figure 3.14. Under normal condition (low eIF2alpha phosphorylation), the uORF2 *GADD34* acts as a barrier for scanning ribosomes. The “barrier” feature of the uORF2 depends on the conserved size of the uORF2, but not on its amino acid sequence (Figure 3.14 top). Increase in basal translation of the main ORF when the uORF2 is truncated, and the presence of the C+G rich intercistronic space that possibly promotes ribosomal dissociation all support the possibility that the basal repression is due to inhibition of reinitiation. During ER stress, the uORF2 still has to be present and translated to allow ribosomes to initiate at the *GADD34* AUG (Figure 3.14 bottom), as the mutant without the uORF2 can no longer be translationally activated during eIF2alpha phosphorylation (Figures 2.8 and 3.10B). It is possible that translation of the uORF2 to synthesize polypeptides at certain length is
**Figure 3.14  Summary of \textit{GADD34} Translational Regulation via Its 5’ UTR**

Under normal condition, the \textit{GADD34} uORF2 acts as a barrier, preventing ribosomes from reaching the \textit{GADD34} AUG. This inhibition is dependent on the size of the uORF2, but not the amino acid sequence of the peptide produced from the uORF2. Under ER stress, basal repression is overcome by translation of the uORF2 which may recruit factors that now allow ribosomes to initiate at the \textit{GADD34} AUG.
required for recruitment of factors that may be expressed specifically during eIF2alpha phosphorylation. These factors may allow ribosomes to overcome the inhibitory elements that repress the basal \textit{GADD34} expression, and to initiate at the \textit{GADD34} AUG.
CHAPTER IV

Development of *in vitro* Systems to Study GADD34 uORF Regulation

4.1 Introduction

4.1.1 Mechanisms of Inhibition of Cap-Dependent Translation

In the previous chapters, characterization of GADD34 uORF2-mediated translational regulation was accomplished by using *in vivo* approaches. In the current chapter, we describe development of *in vitro* translation systems which will help us better understand the mechanism of GADD34 translation. The objective is to elucidate how ribosomes can bypass the uORF in the GADD34 5’ UTR to initiate the main ORF under conditions when overall translation is compromised. There are several advantages of developing and employing an *in vitro* assay system, as they allow us 1) to manipulate and target specific steps during translational initiation, and 2) to monitor the movement of translating ribosomes on reporter mRNAs.

Since GADD34 is translationally upregulated under conditions when overall translation is shut off, the availability and integrity of various factors involved at different stages of translational initiation can be manipulated *in vitro* to monitor regulation of GADD34 translation. My data has shown that GADD34 can be translated during eIF2alpha phosphorylation. However, it is possible that other conditions may allow preferential translation of GADD34. Two key events required for efficient cap-dependent translation are 1) recruitment of TC and 2) formation of eIF4F at the 5’ cap. We propose
to test whether \textit{GADD34} translation is compromised when these events are inhibited in an \textit{in vitro} translation assay.

A key factor that is often regulated for translational control in general is eIF4G, an integral scaffold protein for cap-dependent initiation. eIF4Gs are a common target of proteolysis by various viral proteases upon infection, resulting in inhibition of host cap-dependent translation initiation (Glaser et al., 2003). Picornaviruses produce proteinases that can not only process their translation products, but also cleave host initiation factors, one of which is eIF4Gs (Mohr et al., 2007). Specifically, eIF4Gs are cleaved by the leader proteinase (L\textsuperscript{pro}) of foot and mouth disease virus (FMDV) and by the 2A proteinase (2A\textsuperscript{pro}) of human rhinoviruses (HRVs) and enteroviruses such as polioviruses and coxsackieviruses (Lloyd, 2006; Mohr et al., 2007). Cleavage of eIF4G at its amino terminal eIF4E binding domain results in failure of eIF4F formation at the 5’ cap of host transcripts (Lloyd, 2006). In contrast, the viral IRES can still recruit the carboxy-terminal domain of eIF4G, which engages eIF4A and eIF3 (Lloyd, 2006). Therefore, expression of viral eIF4G proteinases in \textit{in vitro} translation system allows us to recapitulate the cellular environment in which eIF4F formation is disrupted, and it might be possible that the uORFs play a regulatory role to upregulate translation of \textit{GADD34} under such conditions.

In addition to eIF4G, another initiation factor that is heavily targeted for regulation of overall translation is eIF4E, a cap binding protein. The availability of eIF4E for cap-dependent translation initiation is largely controlled by 4E-BPs, eIF4E binding proteins (Raught and Gingras, 2007). 4E-BP and eIF4G compete for the same binding site on eIF4E. Therefore, 4E-BP binding can sequester eIF4E from eIF4G, thus disrupting eIF4F
formation and inhibiting cap-dependent translation (Svitkin et al., 2005). The activity of 4E-BP is regulated by its phosphorylation state. Hypophosphorylated 4E-BP can bind to eIF4E while hyperphosphorylated 4E-BPs do not (Figure 4.1) (Pause et al., 1994; Raught and Gingras, 2007). Several physiological stimuli can regulate the phosphorylation state of 4E-BP to control translation (Raught and Gingras, 2007). Specifically, 4E-BP is known to be phosphorylated when cells are stimulated with hormones, mitogens, and growth factors, thus increasing overall translation rates (Gingras et al., 1999; Sonenberg and Gingras, 1998). mTOR (mammalian target of rapamycin) has been identified to be a key kinase for 4E-BP (Figure 4.1) (Brunn et al., 1997). In contrast, dephosphorylation of 4E-BP is mediated by protein phosphatase 2A in response to nutrient deprivation or viral infections (Figure 4.1) (Gingras et al., 1996; Peterson et al., 1999; Sonenberg and Gingras, 1998). Overexpression of 4E-BP could also lead to inhibition of overall protein synthesis, as 4E-BP overexpression can reverse the phenotype of transformed cell lines (Rousseau et al., 1996). Since GADD34 is translationally upregulated when translation is inhibited when eIF2alpha is phosphorylated, it will be of considerable interest to test if GADD34 translation is upregulated through its uORFs when eIF4F is disrupted by 4E-BP overexpression in vitro.

4.2 Materials and Methods

4.2.1 Materials
Figure 4.1  Regulation of Cap-Dependent Translation by 4E-BP, a eIF4E Binding Protein

eIF4E binding protein 4E-BP exists in two phosphorylation states. When hyperphosphorylated by mTOR, 4E-BP loses affinity for eIF4E which can bind to the 5’ cap of mRNA and recruit eIF4G to form the eIF4F complex, thus activating cap-dependent translation. On the other hand, hypophosphorylated 4E-BP (by protein phosphatase 2A, PP2A) can bind to eIF4E, inhibiting its interaction with eIF4G, and this results in repression of cap-dependent translation.
Dicistronic/monocistronic luciferase reporter constructs containing CrPV IGR IRES cDNA have been previously described (Wilson et al., 2000). NSC119889 was provided by the NCI/DTP Open Chemical Repository. The pCITE-2A plasmid containing the Rhinovirus type 14 2A protease ORF was a generous gift from Dr. Kurt Gustin (University of Arizona College of Medicine). The pAC TAG/HA-4E-BP1 plasmid containing the 4E-BP1 ORF was generously provided from Dr. Martin Bushell (University of Nottingham, UK). Nuclease-treated RRL and TNT RRL were purchased from Promega (Medison, Wisconsin), and non-nuclease-treated RRL was purchased from Green Hectares, USA.

4.2.2 In vitro Transcription

The dicistronic reporter plasmids containing a T7 promoter and Renilla and Firefly ORFs were linearized by restriction digest with XbaI at 37 °C overnight. The linearized plasmids were purified using QIAquick PCR purification kit (QIAGEN). Subsequently, RNAs were synthesized using bacteriophage T7 RNA polymerase. The reactions were carried out at 37 °C for 2 hr in the presence of 1 X RiboMAX transcription buffer (400 mM HEPES-KOH (pH 7.5), 120 mM MgCl₂, 10 mM spermidine, and 200 mM DTT). The DNA was eliminated using DNase I at 37 °C for 30 min. The RNA was purified using RNeasy kit (QIAGEN), and its purity and integrity were confirmed by gel analysis.

4.2.3 Toeprinting Analysis

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The primer PrEJ663 (5’-TGCCGGTGGTGAGAATTCCAG-3’) was radiolabeled with \(\gamma^{32}\)P]ATP by PNK. 500 ng of mouse GADD34 5’ UTR-YFP or 1&2KO mouse GADD34 5’ UTR-YFP RNAs were annealed with primer in 40 mM Tris (pH 7.5) and 0.2 mM EDTA by slow cooling from 65 °C to 37 °C. The RNA was then incubated in nuclease-treated RRL (Promega) with 25 μM amino acid mix (Promega) and 0.5 mg/ml cycloheximide for 15 min at 30 °C. To detect the location of the ribosome, primer extension assay using AMV reverse transcriptase (Promega) was used. cDNA products were purified by phenol/chloroform extraction followed by EtOH precipitation, and analyzed by PAGE (8 M urea/6% (w/v) polyacrylamide gel). Gels were dried and analyzed by phosphorimager analysis.

4.2.4 Reconstitution of eIF2alpha Phosphorylation in Untreated RRL

Untreated RRL (Green Hectares, USA) was supplemented with 25 μM haemin (Fluka), 25 μg/ml creatine phophokinase (Sigma-Aldrich Co.), 5 mg/ml creatine phosphate (Fluka), 50 μg/ml bovine bulk tRNA (Novagen), and 3 mM D-glucose (Sigma-Aldrich Co.). Untreated RRL without hemin was incubated at 30 °C to induce eIF2alpha phosphorylation in vitro. In parallel, the haemin-containing RRL supplemented with 2-aminopurine (2AP) (Sigma-Aldrich Co.) was used to inhibit eIF2alpha phosphorylation. eIF2alpha phosphorylation at 60 min was evaluated by Western blotting. Reporter YFP RNAs were incubated in the lysates containing 100 mM
potassium acetate at 30 °C for 60 min, and the YFP protein was measured by Western blotting.

4.2.5 *In vitro* Translation Assay

0.4 µg of uncapped dicistronic/monocistronic reporter RNAs containing CrPV IGR IRES, WT or mutant 1&2 KO mouse *GADD34* 5’ UTR were added to nuclease-treated RRL (Promega) with 154 mM potassium acetate, and the reactions were carried out at 30 °C for 1 hr. For drug treatment, NSC119889 was added to RRL at 30 °C for 10 min prior to addition of the RNAs. To express 2A pro in the translation system, 0.4 µg of purified 2A pro RNA was added to nuclease-treated RRL for 30 min at 30 °C prior to addition of the reporter RNAs, and the reactions were incubated for 1 hr. For 4E-BP expression, a final concentration of 0.1 µg/µl of the 4E-BP1-expressing plasmid was incubated in a TNT RRL (Promega) for 30 min at 30 °C prior to addition of the reporter RNAs. Renilla and Firefly luciferase activities were measured by a Stop and Glo kit (Promega). Luminescence was measured using a luminometer (Berthold Technologies). Alternatively, luciferase proteins were measured by [35S]methionine incorporation, and were analyzed by SDS-PAGE and autoradiography.

4.3 Results

4.3.1 Positioning of Ribosomes on the *GADD34* 5’ UTR *in vitro*
As previously discussed in the Chapter 2, the uORF1 of GADD34 is poorly translated due to its lack of optimal Kozak consensus AUG. Thus, a significant number of ribosomes skip the uORF1 AUG to initiate translation at the downstream uORF2. To determine the location of ribosomes on the GADD34 5' UTR, we performed toeprinting analysis of RNA in nuclease-treated rabbit reticulocyte lysates (RRL) (Figure 4.2). In this assay, the location of the ribosomes on an RNA can be detected by primer extension. Briefly, the position of ribosomes on a given transcript can be determined by synthesizing cDNA products from a radiolabeled primer via reverse transcription. Reverse transcription will continue until the bound ribosomes are encountered, thus producing a truncated cDNA which is then separated by denaturing PAGE. The YFP reporter RNAs containing WT (mGADD34-YFP) and 1&2 KO mouse GADD34 5' UTR (Mutant 1&2 KO mGADD34-YFP) were added to the RRL in the presence of cycloheximide. Cycloheximide inhibits the translocation of the ribosomes, and therefore, this assay will monitor the location of ribosome assembly during translational initiation. Normally, addition of cycloheximide is known to produce distinct toeprints at +15-16 away from the first nucleotide (+1) of the AUG in the P-site by reverse transcription with a radiolabeled primer (Pestova and Hellen, 2003). For mGADD34-YFP RNA, we observed toeprints at CG_{94-95} and UC_{195-196} (16-17 nucleotides downstream of the uORF1 and 2 AUGs, respectively) from ribosomes that are stalled at the AUGs of uORF1 and 2 (Figure 4.2, see filled arrow heads in Lane 1). This confirms that the uORFs can block ribosomes from reaching the main ORF, and is consistent with the previous observation that these uORFs can repress translation of the main ORF. When both uORFs are knocked out (Mutant 1&2 KO mGADD34-YFP), the previous toeprints at CG_{94-95} and
The positions of the ribosomes on the 5' UTR of mouse GADD34 were visualized by reverse transcription. Mouse GADD34 5'UTR-YFP (1) or mutant 1&2 KO mouse GADD34 5'UTR-YFP (2) was added to RRL with radiolabeled PrEJ663 to allow the ribosomes to bind the transcripts in the presence of 0.5 mg/ml cycloheximide. Subsequently, primer extension was performed using AMV reverse transcriptase, and the cDNA products were analyzed by PAGE (8M urea/6% (w/v) polyacrylamide gel). The filled arrowheads in lane 1 represent the ribosomes positioned at the AUGs of uORFs, whereas a blank arrowhead in lane 2 represent the ribosomes at the AUG of the main YFP ORF. The positions of the nucleotides are according to the mouse GADD34 mRNA sequence (NM_008654) from GenBank™.
UC_{195-196} were missing, but new toeprints now appeared at GA_{299-300} (16-17 nucleotides downstream of the \textit{YFP ORF AUG}), indicating that most ribosomes were now stalled at the AUG of the main \textit{YFP ORF} (Figure 4.2, see empty arrow head in Lane 2). This suggests that the scanning ribosomes now initiate at the \textit{YFP ORF AUG}, and is in agreement with the previous findings in which 1&2 KO mutant exhibited much higher basal translation than the WT.

4.3.2 Establishment of eIF2alpha Phosphorylation \textit{in vitro}

Since \textit{GADD34} is translated during ER stress when eIF2 is phosphorylated, we wished to establish an \textit{in vitro} system that can recapitulate this condition. Towards this, polyinosinic:polycytidylic acid (poly I:C), a dsRNA mimic, was initially used to induce eIF2alpha phosphorylation in non-nuclease-treated RRL via PKR. However, this proved to be inconsistent and not reproducible in our hands. Instead, I found that incubation of the non-nuclease-treated RRL at 30 °C for 60 minutes resulted in a significant increase in eIF2alpha phosphorylation. In contrast, RRL supplemented with hemin, an inhibitor of HRI activation, and 2-Aminopurine (2AP), an adenine isomer that binds and inhibits HRI and PKR activity, resulted in dephosphorylated eIF2alpha (Figure 4.3) (Chen and London, 1995; Farrell et al., 1977; Jarrous et al., 1996; Thomis and Samuel, 1993).

Despite the ability to selectively induce eIF2alpha phosphorylation in the non-nuclease-treated RRL, we could not detect translation products (by \textsuperscript{35}S radiolabeling) when reporter \textit{YFP RNAs} were added (data not shown), indicating that the lysates were not optimized for translation. Moreover, translation of globin mRNAs, which is the most
Figure 4.3  Induction of eIF2alpha Phosphorylation in the Non-Nuclease Treated RRL

Western blots of phosphorylated eIF2alpha (upper panel) and total eIF2alpha (lower panel) from non-nuclease treated RRL with or without hemin and 2AP. The RRL was incubated at 30 °C for 60 min without any RNA before subjected to Western blotting analysis by LI-COR Odyssey imaging system (Lincoln, NE).
abundant endogenous RNA in RRL, was not observed. Thus, further optimization is required to faithfully recapitulate eIF2alpha phosphorylation \textit{in vitro}.

4.3.3 Inhibition of Overall Translation by NSC119889 in RRL \textit{in vitro}

Since we could not recapitulate eIF2alpha phosphorylation in the RRL, we turned to another approach to simulate its inhibitory effect. The drug, NSC119889, was found to inhibit TC formation in various cell-free translation extracts such as Krebs-2, RRL, and HeLa extracts (Figure 4.4) (Novac et al., 2004; Robert et al., 2006). Thus, the effect of NSC119889 inhibits eIF2 activity, and is quite similar to the conditions when eIF2alpha is phosphorylated. Therefore, NSC119889 could serve as a suitable alternative to recapitulate cellular conditions where eIF2alpha is phosphorylated \textit{in vitro}. Towards optimization of this system, we first tested the effect of this drug on scanning and CrPV-IRES dependent translation. The CrPV IRES can recruit ribosomes in the absence of initiation factors (Jan and Sarnow, 2002), and therefore, serves as a suitable control which should be translated during NSC119889 drug treatment. We incubated increasing amounts of NSC119889 in RRL for 10 minutes before adding a dicistronic reporter RNA in which Renilla and Firefly luciferase ORFs are separated by the CrPV IRES (Figure 4.5A). Addition of NSC119889 inhibited cap-dependent translation, as previously found (Figure 4.5B) (Novac et al., 2004). However, translation of Firefly luciferase remained resistant to the drug treatment at 10 and 25 \(\mu\)M, and even at 50 \(\mu\)M, its translation was only inhibited by approximately 20\% from the basal level, while this concentration of NSC119889 almost completely abolished cap-dependent translation (Figure 4.5B). This
Figure 4.4  Inhibition of Ternary Complex Formation by NSC119889

(A) Chemical structure of NSC119889.
(B) Proposed mechanisms of inhibition of ternary complex formation by NSC119889 (Robert et al., 2006).
Figure 4.5  Inhibition of Cap-Dependent Translation by NSC119889

(A) Schematic of the dicistronic reporter RNA containing an IRES element flanked by upstream Renilla luciferase ORF and downstream Firefly luciferase ORF.

(B) Translation via CrPV IGR IRES in RRL treated with NSC119889. Dicistronic reporter RNA with CrPV IGR IRES was incubated in nuclease treated RRL with 10, 25, 50 µM of NSC119889 at 30 ºC for 60 min. Following incubation, 2 µl of the reactions was subjected to luciferase assay to measure Renilla (black) and Firefly (white) luciferase activities. % Activity was normalized to the activity without NSC119889 treatment. The experiment was performed in triplicate, and the results were averaged. Error bars represent ± standard deviation.
result confirmed that the cap-dependent translation in RRL can selectively inhibited by using NSC199889. Since GADD34 is translationally regulated under eIF2alpha phosphorylation which limits TC availability, we predict that the GADD34 5’ UTR can mediate translation in presence of NSC119889 in vitro.

4.3.4 Inhibition of Overall Translation by Expression of Human Rhinovirus Type 2 2A\textsuperscript{pro} in RRL in vitro

To develop a system that inhibits eIF4F complex formation, we have taken two approaches. In one, we have compromised eIF4G, and in the other, we have inhibited the availability of eIF4E. In the first approach, we employed the 2A\textsuperscript{pro} of human rhinoviruses in our in vitro system. The 2A\textsuperscript{pro} of human rhinoviruses specifically cleaves eIF4G at its amino terminal domain, thereby inhibiting recruitment of eIF4G to the 5’cap of transcripts and overall translation (Haghighat et al., 1996; Mohr et al., 2007). To first establish this assay, we again monitored CrPV IRES-driven translation using the dicistronic reporter RNA (Figure 4.6). To express 2A\textsuperscript{pro}, we added an in vitro synthesized RNA encoding the 2A\textsuperscript{pro} to the nuclease-treated RRL for 30 min prior to addition of the dicistronic reporter RNA. 2A\textsuperscript{pro} expression was detected after 30 minutes, as shown by radiolabeled bands (16 kDa) on SDS-PAGE (0 time point in Figure 4.6A). Subsequently, we added the dicistronic RNAs and took aliquots at 0, 30 and 60 minutes after incubation. When 2A\textsuperscript{pro} was added, we found that the Renilla luciferase expression is inhibited, indicating that as expected, cap-dependent translation is shut off with 2A\textsuperscript{pro} expression (Figure 4.6). Interestingly, Firefly luciferase translation directed by the CrPV IGR IRES
Figure 4.6  **Inhibition of Cap-Dependent Translation by 2A<sup>pro</sup>**

(A) Autoradiogram showing [³⁵S] radiolabeled protein products from *in vitro* translation reactions containing reporter RNAs with or without 2A<sup>pro</sup> expression. 2A<sup>pro</sup> RNA was incubated in nuclease treated RRL (Promega) for 30 min at 30 °C prior to addition of the reporter RNAs. Following RNA addition, samples were taken at respective time points, and separated on 15% SDS-PAGE.

(B) Quantification of the fold luciferase activities under 2A<sup>pro</sup> expression as shown in (A) measured by dual luciferase luminescence assay. The fold changes of Renilla (black) and Firefly (white) luciferase activities at respective time points were normalized to the sample without 2A<sup>pro</sup> expression which was set as 1. The experiment was performed in triplicate, and the results were averaged. Error bars represent ± standard deviation.
remained active under 2Apro expression (Figure 4.6). Hence, we have successfully established a system where eIF4G can be cleaved 2Apro, and can now test if GADD34 can be translated when eIF4F formation is compromised by eIF4G cleavage.

4.3.5 Inhibition of Overall Translation by Overexpression of 4E-BP in RRL in vitro

The second strategy is to disrupt eIF4F formation by overexpressing 4E-BPs in vitro. As previously described, 4E-BP proteins compete for eIF4E against eIF4G, thus negatively regulating cap-dependent translation (Raught and Gingras, 2007). Toward creating this in vitro system, we incubated a plasmid containing 4E-BP1 in a coupled transcription-translation RRL (TNT-RRL). As shown in autoradiogram in Figure 4.7A, radiolabeled 4E-BP protein was readily detected by [35S]methionine in TNT-RRL after 30 minutes. Then, we added the dicistronic reporter RNA containing the CrPV IGR IRES to test efficiency of translation in this system. In the absence of 4E-BP overexpression, cap-dependent translation of Renilla luciferase ORF was active at both 30 and 60 minute time points, while translation of Firefly luciferase ORF was also detected, but at lower efficiency (Figure 4.7A). On the other hand, when 4E-BP was expressed, Renilla luciferase expression was drastically inhibited at both 30 and 60 min time points, confirming that overexpression of 4E-BP indeed resulted in a decrease in overall cap-dependent translation (Figure 4.7). Interestingly, there was a notable increase in Firefly luciferase expression (by approximately 2 to 4 fold) with 4E-BP overexpression,
Figure 4.7  Inhibition of Cap-Dependent Translation by 4E-BP Overexpression

(A) Autoradiogram showing $^{35}$S radiolabeled protein products from tin vitro translation reactions containing reporter RNAs with or without 4E-BP overexpression. The plasmid expressing 4E-BP1 was incubated in TNT RRL (Promega) for 30 min at 30 °C prior to addition of the reporter RNAs. Following RNA addition, samples were taken at respective time points, and separated on 15% SDS-PAGE .

(B) Quantification of the fold luciferase activities under 4E-BP overexpression as shown in (A) measured by dual luciferase luminescence assay. The fold changes of Renilla (black) and Firefly (white) luciferase activities at respective time points were normalized to the sample without 4E-BP overexpression of which the fold was given 1. The experiment was performed in triplicate, and the results were averaged. Error bars represent ± standard deviation.
indicating that CrPV IGR IRES-mediated cap-independent translation is stimulated under such conditions (Figure 4.7).

Now that we had a system that inhibits eIF4E activity, we wished to ask whether the GADD34 5’ UTR can mediate translation when 4E-BPs are overexpressed. Here, a monocistronic Firefly luciferase reporter RNAs containing the 5’ UTR of mouse GADD34 (both WT and 1&2 KO mutant) were incubated in TNT-RRL in which 4E-BP was overexpressed (Figure 4.8A and B). As a positive control, a monocistronic reporter RNA with CrPV IGR IRES was used in parallel (Figure 4.8C), and a monocistronic Renilla luciferase reporter RNA without any regulatory element was added as a negative control. When 4E-BP was overexpressed, the Renilla luciferase activity was abolished by nearly 90%, indicating that cap-dependent translation was inhibited by the overexpression (Figure 4.8). As shown previously, translation mediated by the CrPV IRES remained active (Figures 4.7 and 4.8C). Surprisingly, translation of the reporter RNA containing mouse GADD34 5’ UTR with the intact uORFs was resistant to 4E-BP expression (Figure 4.8A). In contrast, translation of the Mutant 1&2 KO mGADD34-FF RNA was inhibited by 50% after 60 minutes (Figure 4.8B). These preliminary results suggest that the GADD34 uORFs can direct translation when eIF4F formation is compromised by 4E-BP expression.

### 4.4 Discussion

In order to further characterize translational regulation of GADD34 via 5’ UTR elements, it will be useful to develop an in vitro translational system that simulates the conditions when GADD34 is translated. In vitro approaches offer several advantages in
Figure 4.8  Translation via Mouse GADD34 5' UTR during 4E-BP Overexpression in vitro

Quantification of the fold luciferase activities when 4E-BP is expressed. Luciferase was measured by luciferase luminescence assay. Following overexpression of 4E-BP1 in TNT RRL for 30 min, the monocistronic reporter RNAs containing WT mouse GADD34 5’ UTR (A), mutant 1&2 KO mouse GADD34 5’ UTR (B), or CrPV IGR IRES (C) was added to the translation system. In all reactions, the monocistronic Renilla luciferase RNA was also added as a control. The fold changes of Renilla (black) and Firefly (white) luciferase activities at the respective time points were normalized to the sample without 4E-BP overexpression which was set as 1.
studying mechanisms of translation over *in vivo* approaches. Firstly, the exact position of ribosomes on an mRNA can be monitored by using a toeprinting assay. In this chapter, we show that the ribosomes are located at both uORF1 and 2 AUGs of *GADD34*, but not at the AUG of the main ORF (Figure 4.3 Lane 1). When the AUGs of *GADD34* uORFs were knocked out by mutagenesis, the ribosomes were positioned at the AUG of the downstream *YFP* ORF (Figure 4.3 Lane 2). These results confirm that the uORFs basally block scanning ribosomes from reaching the main ORF. Moreover, because we observe ribosomes at both AUG codons of uORF1 and uORF2, this indicates that ribosomes are bypassing uORF1, which is consistent with our previous data that the uORF1 is dispensable for blocking scanning ribosomes (Chapter 2). We also noticed additional toeprints within the 5′ UTR that do not correspond to ribosome positioning at an AUG codon (Figure 4.3). It is possible that secondary structures present along the 5′ UTR of *GADD34* could potentially hamper movement of the ribosomes, and this might result in appearance of additional toeprints. Although it is unclear why the ribosomes are positioned at other parts of *GADD34* 5′ UTR in addition to the AUGs, it is clear that the scanning ribosomes initiate at both AUGs of the *GADD34* uORF1 and 2, blocking access to the AUG of the downstream main ORF.

Another advantage of the *in vitro* system is that specific steps of translation can be targeted and inhibited. Since *GADD34* is translationally upregulated through its 5′ UTR uORFs when eIF2alpha is phosphorylated during ER stress in cells, it was thought that recapitulating eIF2alpha phosphorylation *in vitro* would be useful to study uORF-mediated translational regulation. To achieve this, crude non-micrococcal nuclease-treated RRL was used to reconstitute a translational system as opposed to the more
commonly used nuclease-treated RRL. The difference between these two types of RRL is that a nuclease-treated RRL has already been optimized for maximal translation activity by micrococcal nuclease treatment and addition of hemin, an inhibitor of eIF2alpha kinase HRI. Therefore, in order to induce eIF2alpha phosphorylation in vitro, hemin had to be excluded from the crude RRL that was reconstituted with other required components. Initially, poly I:C, a dsRNA mimic, was used to activate the kinase PKR in the RRL, as this is a commonly used method to induce eIF2alpha phosphorylation (Jackson and Hunt, 1983). Unfortunately, poly I:C failed to induce a significant increase in eIF2alpha phosphorylation in the reconstituted RRL in the current investigation. Interestingly, however, incubation of the lysate at 30 °C for up to 60 min resulted in noticeable eIF2alpha phosphorylation compared to the lysate that was supplemented with hemin and 2AP, an inhibitor of PKR (Figure 4.4). This observation correlates with a suggestion that freeze-thawing and prolonged incubation of the lysate at higher temperatures than its storage temperature (-20 to -80 °C) should be avoided to prevent loss of translational efficiency, as these could very well lead to phosphorylation of eIF2alpha in the extract. Although phosphorylation of eIF2alpha could selectively be induced in vitro, translational efficiency in this lysate was poor, as translation of the reporter RNAs was not detected. In fact, by [35S] labelling, translation of globin RNA, the most abundant transcript in RRL, was also absent, indicating that the current RRL system requires further optimization for its translational capacity in addition to eIF2alpha phosphorylation. It is highly possible that the concentration of salts such as K+ and Mg2+ in the reaction greatly affects translation in in vitro extracts. Therefore, it might be
worthwhile to refer to previous literature where the cell-free translational system was successfully reconstituted using the untreated RRL (Soto Rifo et al., 2007).

In order to recapitulate eIF2alpha phosphorylation through other means in vitro, a compound called NSC119889 was used. NSC119889 was previously found to inhibit cap-dependent translation by disrupting TC formation (Robert et al., 2006). Incubation of NSC119889 at varying concentrations in the nuclease-treated RRL with the dicistronic reporter RNA showed that cap-dependent translation was successfully inhibited by the drug treatment (Figure 4.5). At the same time, cap-independent translation mediated by CrPV IGR IRES remained active under NSC119889 in vitro (Figure 4.5). This is in agreement with previous data which showed that the CrPV IGR IRES was functional when eIF2alpha was phosphorylated in vivo, and its translation was active in Kreb-2 extract (Fernandez et al., 2002; Robert et al., 2006). Therefore, the cellular condition where TC availability is limited by eIF2alpha phosphorylation under stress was faithfully mimicked in vitro. NSC119889 in the in vitro translation system could potentially serve as a useful tool to ask a question whether TC availability has a direct impact on translational regulation of GADD34 via its 5’ UTR uORFs.

In addition to eIF2alpha phosphorylation, we also wished to simulate inhibition of overall cap-dependent translation by disrupting other factors in the process in vitro. This was achieved through negative regulation of eIF4F formation at the 5’cap of mRNAs via 1) expression of Human Rhinovirus Type 2 2Apro and 2) overexpression of eIF4E binding protein 4E-BP. Specifically, 2Apro inhibits cap-dependent translation by cleaving eIF4GI, while 4E-BP overexpression prevents interaction between eIF4G and eIF4E, thus impairing formation of eIF4F (Mohr et al., 2007; Svitkin et al., 2005). Again, in our in
*vitro* translation system, cap-dependent translation was inhibited by these treatments, while the CrPV IGR IRES, which does not require any initiation factors to recruit ribosomes, was found to be still active (Figure 4.6 and 4.7). When *GADD34* was tested to see how its 5’ UTR regulates *GADD34* translation under these circumstances, it was surprising to notice that in contrast to the drastic inhibition in translation of monocistronic *Renilla* luciferase reporter RNAs which lack any regulatory elements in their 5’ UTR, the RNA with the WT *GADD34* 5’ UTR exhibited resistance to 4E-BP overexpression. However, under the same condition, the mutant 1&2 KO *GADD34* 5’ UTR was less active in translation than WT (Figure 4.8A and B). Although the data are preliminary, this finding warrants further investigation. It would also be interesting to see how translational regulation of endogenous *GADD34* is affected under hypophosphorylation of 4E-BPs by rapamycin which inhibits mTOR, a kinase that phosphorylates 4E-BPs. Furthermore, these *in vitro* systems have much potential to be used to study other transcripts with uORFs as well as to screen for novel genes that can selectively be translated under inhibition of overall protein synthesis.
CHAPTER V
Summary and Future Studies

5.1 Summary

In the current study, we present a novel mechanism for translation of GADD34 mRNA via its uORF2 during ER stress. GADD34 is a key player in the negative feedback loop of the ER stress pathway to mediate dephosphorylation of eIF2alpha, thus leading to an overall recovery of translation. Previous reports have shown that GADD34 expression is due to transcriptional induction during ER stress (Ma and Hendershot, 2003; Novoa et al., 2001). Here, we have demonstrated that GADD34 is also translationally upregulated, which is likely important for maximal expression of GADD34 during ER stress. First, the endogenous GADD34 mRNAs are associated with polysomes under ER stress. Second, in the absence of transcriptional activation during ER stress, GADD34 is still produced from the pre-existing GADD34 mRNAs via translational upregulation. Translational regulation of GADD34 is dependent on the uORFs present in its 5’ UTR, as a reporter RNA with the GADD34 uORFs is still associated with and translated by polysomes under ER stress. In particular, translation of the uORF2 is required to repress basal translation of GADD34, while the uORF1 is dispensable and is not recognized by scanning ribosomes via leaky scanning.

By polysomal analysis and transient transfection, we showed that the GADD34 5’ UTR without the uORF1 was sufficient in regulating translation under ER stress. This regulation via the uORF2 was not dependent on its conserved amino acid or nucleotide
sequence, but on its conserved 26 codon-long size. The shortened uORF2 failed to repress translation of the main ORF under normal conditions, and upon ER stress, it could not mediate translational upregulation of the main ORF. The sequence conservation of intercistronic space between the uORF2 and \textit{GADD34} was also tested. Lengthening and changing cytosines to guanines in the intercistronic space did not affect its translational regulation. Based on the results, we propose that translation of the \textit{GADD34} uORF2 prevents the access of the ribosomes to the \textit{GADD34} AUG by preferentially initiating translation at its own AUG, and the size of the uORF2 is crucial in this repression. During ER stress, the ribosomes can initiate at the \textit{GADD34} AUG although the uORF2 translation is still required. It is possible that translation of the uORF2 to produce a polypeptide of certain length might be required to recruit/mediate factors that help the ribosomes overcome inhibitory blocks under basal condition, and further studies are necessary to explore this possibility.

We also developed \textit{in vitro} translational systems that simulate inhibition of cap-dependent translation in cells. The \textit{in vitro} systems offer several advantages such as 1) movement of ribosomes on a given transcript can be monitored and 2) translation can be manipulated in our favour by using drugs or overexpressing certain factors. These \textit{in vitro} systems inhibited cap-dependent translation by blocking TC and eIF4F formation. The former was achieved by using the drug NSC119889, while the latter was done by expressing 4E-BPs or 2A\textsuperscript{pro}. Therefore, \textit{GADD34} can be tested in these \textit{in vitro} systems to ask whether it is translated under these conditions.

In conclusion, the results in this thesis suggest that expression of \textit{GADD34} is tightly regulated at both transcriptional and translational levels. Indeed, overexpression of
GADD34 was previously observed to lead to disruptions in normal apoptotic pathways (Adler et al., 1999) and glucose homeostasis (Oyadomari et al., 2008). We showed that the uORF2 present in the 5’ UTR of GADD34 is required to maintain its translational regulation under basal and stress conditions. Specifically, the uORF2 represses the basal translation, but promotes translation of the main ORF during ER stress. How the ribosomes can initiate at the AUG of the main ORF under this condition is still unclear, and there are possible models by which the ribosomes accomplish this (Figure 5.1). First, as previously mentioned, translation of the uORF2 under ER stress could allow the ribosomes to resume scanning and reinitiate at the downstream GADD34 AUG. It is possible that factors that are specifically produced or available in greater abundance during ER stress might promote this by interacting with the GC rich intercistronic space of GADD34 (Figure 5.1A). Also, translation of the uORF2 under ER stress could signal for ribosomal shunting, in which the ribosomes on the uORF2 now “jump” and “land” at the GADD34 AUG to initiate translation (Figure 5.2B). This example is shown in cauliflower mosaic virus (CaMV), where the ribosomes shunt following translation of its short uORF, and land at the major viral ORF downstream (Mohr et al., 2007). In the case of GADD34, ER stress might be a cue to elicit this shunting mechanism somehow, as the uORF2 is translated at under both basal and stress conditions (Figure 5.2B).

Ultimately, understanding this novel mechanism of GADD34 can open many doors for research on translational control by uORFs. To date, only a handful of uORFs-mediated translational mechanism has been characterized. The most notable ones are
How can the ribosomes initiate at the *GADD34* AUG under ER stress?

**Figure 5.1  Possible Models of *GADD34* Translation during ER Stress**

During ER stress, the *GADD34* is translated by ribosomes. There are several models for this phenomenon. It is possible that the polypeptide from the uORF2 translation might be involved in recruitment of factor(s) which are specifically expressed under ER stress, and this interaction may somehow help the ribosomes reinitiate at the *GADD34* AUG (A). Also, ER stress could elicit ribosome shunting, so that the ribosomes “jump” following translating of the uORF2, and “land” at the AUG of *GADD34* to initiate translation.
found in ATF4 (Lu et al., 2004) and GCN4 (Mueller and Hinnebusch, 1986), while a single uORF of the transcription factors C/EBPalpha and beta also regulates translation under eIF2alpha phosphorylation (Calkhoven et al., 2000; Oyadomari et al., 2008). Given that a number of uORF-containing RNAs have been identified to influence expression of a vast number of genes (Calvo et al., 2009), the GADD34 mechanism could potentially serve as a model to study how other 5’ UTR uORF-containing RNAs are regulated under cellular stress.

5.2 Future Studies

Despite the results presented in this thesis, the exact mechanism of translational regulation via the GADD34 uORF2 remains unclear. Therefore, some additional experiments would greatly help us better characterize and test the current model of the GADD34 translational regulation.

Firstly, the inhibitory effect of the high C+G content in the intercistronic space needs to be tested on the movement of the ribosomes. We speculate that basally, the ribosomes could dissociate after translating the uORF2 due to the C+G rich context of the termination codon, thus failing to reach the AUG of the main ORF. This may provide an explanation for the translational repression of GADD34 observed under normal condition. There is already precedence that the C+G content around the stop codon of the inhibitory uORF in yeast GCN4 promotes dissociation of the ribosomes, preventing efficient reinitiation at the GCN4 AUG under non-starving condition (Grant and Hinnebusch, 1994). In the thesis, we mutated every cytosine to guanine in the intercistronic region to
ask whether sequence identity in this region is significant. However, the C+G content remained unchanged by this mutation, and this may explain for the basal repression observed in this mutant, as the ribosomes might still dissociate from the transcript following translation of the uORF2. In order to test this possibility, the sequence of the intercistronic distance can be completely randomized by site directed mutagenesis to change the C+G content within this region. If a higher basal expression of the main ORF is observed with mutation, we can safely conclude that the conserved high C+G content in the intercistronic region acts the same way as in GCN4 to keep translation low in the absence of eIF2alpha phosphorylation.

We previously found that the truncating the uORF2 from 26 to 15 codons allowed a dramatic increase in basal translation of the main ORF. This phenomenon may suggest that the ribosomes can reinitiate at the downstream AUG more efficiently after translating the shorter uORF2, as it has been well known that efficiency of reinitiation at a downstream ORF is inversely related to the length of an upstream ORF translated (Kozak, 2001). However, our truncation mutant was created by introducing a premature stop codon within the uORF2, and this inevitably changed the size of the intercistronic space and the termination codon context which all might have contributed towards enhancement of reinitiation. Therefore, it is necessary to test if changing only the length of the uORF2 bears any effect on the translational regulation. To do this, the uORF2 AUG will need to be moved further downstream rather than moving its stop codon upstream. This way, the length of the intercistronic space and the stop codon context are preserved, while the size of the uORF2 is shortened. The high basal translation of the main ORF with this mutation would argue against the possible inhibitory function of the
C+G rich stop codon context as a strong promoter of ribosome dissociation, and the basal repression is mediated through an alternate mechanism.

Lastly, it might be worthwhile to revisit phosphorylation of eIF2alpha in the untreated lysate for studying translational regulation of GADD34 in vitro. As previously mentioned, an in vitro translational system is a useful tool that allows us to monitor the movement of ribosomes on a given transcript, and to manipulate translation by using drugs or modifying expression/activity of certain factors. Since GADD34 is expressed via its 5’ UTR during eIF2alpha phosphorylation under ER stress, it would be important to test its regulation in vitro when eIF2alpha is phosphorylated. In this thesis, we demonstrated that our in vitro system was able to phosphorylate eIF2alpha, yet was translationally inactive. This in vitro system can be further optimized according to the previous literature in which the untreated lysate was successfully reconstituted to be translationally competent (Soto Rifo et al., 2007). In this system, GADD34 5’ UTR containing reporter RNAs can be tested to examine the movement and positions of the ribosomes on the transcripts under eIF2alpha phosphorylation. The in vitro system also allows us to identify any factors that may be associated with the ribosomes on the GADD34 5’ UTR under eIF2alpha phosphorylation. These factors may be key to mediate translational regulation of GADD34 via its 5’ UTR under ER stress, and therefore, the use of the in vitro will shed light on the unique translational control mechanism of GADD34.
References


Chou, J., and B. Roizman. 1994. Herpes simplex virus 1 gamma(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. Proc Natl Acad Sci U S A. 91:5247-51.


