

An Upstream Open Reading Frame Regulates Translation of GADD34 during Cellular Stresses That Induce eIF2 α Phosphorylation^{*[5]}

Received for publication, September 2, 2008, and in revised form, December 16, 2008 Published, JBC Papers in Press, January 8, 2009, DOI 10.1074/jbc.M806735200

Yun-Young Lee[‡], Randal C. Cevallos[§], and Eric Jan^{†1}

From the [‡]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada and the [§]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305

Cellular stress such as endoplasmic reticulum stress, hypoxia, and viral infection activates an integrated stress response, which includes the phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α) to inhibit overall protein synthesis. Paradoxically, this leads to translation of a subset of mRNAs, like transcription factor ATF4, which in turn induces transcription of downstream stress-induced genes such as growth arrest DNA-inducible gene 34 (GADD34). GADD34 interacts with protein phosphatase 1 to dephosphorylate eIF2 α , resulting in a negative feedback loop to recover protein synthesis and allow translation of stress-induced transcripts. Here, we show that GADD34 is not only transcriptionally induced but also translationally regulated to ensure maximal expression during eIF2 α phosphorylation. GADD34 mRNAs are preferentially associated with polysomes during eIF2 α phosphorylation, which is mediated by its 5'-untranslated region (5'UTR). The human GADD34 5'UTR contains two non-overlapping upstream open reading frames (uORFs), whereas the mouse version contains two overlapping and out of frame uORFs. Using 5'UTR GADD34 reporter constructs, we show that the downstream uORF mediates repression of basal translation and directs translation during eIF2 α phosphorylation. Furthermore, we show that the upstream uORF is poorly translated and that a proportion of scanning ribosomes bypasses the upstream uORF to recognize the downstream uORF. These findings suggest that GADD34 translation is regulated by a unique 5'UTR uORF mechanism to ensure proper GADD34 expression during eIF2 α phosphorylation. This mechanism may serve as a model for understanding how other 5'UTR uORF-containing mRNAs are regulated during cellular stress.

Phosphorylation of Ser⁵¹ in eIF2 α is a key cellular response to environmental stresses such as hypoxia, endoplasmic reticulum (ER)² stress and viral infection. The stress-induced phos-

phorylation of eIF2 α represses general protein synthesis, which induces the expression of specific genes involved in the stress response (1, 2). Reprogramming of gene expression is vital for cellular survival and can trigger apoptosis, if the stress is severe and prolonged.

In mammals, four distinct eIF2 α kinases have been identified (2). These include protein kinase R, which is activated upon binding to double-stranded RNAs or through the antiviral interferon response (3), the heme-regulated inhibitor, which senses heme availability and responds to oxidative stress (4, 5), the general control nonderepressible-2, which is regulated by amino acid availability (6), and the protein kinase R-like ER kinase, PERK, which is activated in response to an accumulation of unfolded proteins in the ER (7). Although protein kinase R, heme-regulated inhibitor, general control nonderepressible-2, and PERK can all catalyze the phosphorylation of eIF2 α to halt protein synthesis, they do so in response to distinct environmental cues. For instance, the accumulation of unfolded proteins in the ER activates PERK to repress translation to ease the load of unfolded proteins in the ER, whereas translational repression during amino acid depletion is achieved through general control nonderepressible-2 activation, which provides cells sufficient time for recovery from nutrient starvation. The biological importance of each eIF2 α kinase signaling pathway is reflected in their association with several diseases such as diabetes, cancer, neurodegenerative diseases, and viral infections, including hepatitis C virus and cytomegalovirus infections (8–12).

The ternary complex, composed of initiator Met-tRNA_i, GTP, and the heterotrimeric initiation factor eIF2, mediates recognition of the AUG codon by scanning 40 S ribosomal subunits (2, 13). Upon correct basepairing of the codon-anticodon of the Met-tRNA_i, eIF2-GTP is hydrolyzed to eIF2-GDP and subsequently released. Free eIF2-GDP is recycled to the green fluorescent protein-bound form by the guanine nucleotide exchange factor, eIF2B. Phosphorylation of the α subunit of eIF2 α on Ser⁵¹ prevents the exchange of GDP for GTP by sequestering eIF2B, thus lowering the available pool of eIF2-GTP and repressing protein synthesis (14, 15).

Although general protein synthesis is repressed when eIF2 α is phosphorylated, a subset of mRNAs remains actively trans-

tein response; RACE, rapid amplification of cDNA ends; YFP, yellow fluorescent protein; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRES, internal ribosome entry site; C/EBP, CAAT/enhancer-binding protein; GADD34, growth arrest DNA-inducible gene 34.

* This work was funded by a Canadian Institutes for Health Research operating grant (to E. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

¹ To whom correspondence should be addressed: 5457-2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Tel.: 604-827-4226; Fax: 604-822-5227; E-mail: ej@interchange.ubc.ca.

² The abbreviations used are: ER, endoplasmic reticulum; PERK, protein kinase R-like ER kinase; eIF2 α , eukaryotic initiation factor 2 α ; ORF, open reading frame; uORF, upstream ORF; UTR, untranslated region; UPR, unfolded pro-

5' UTR GADD34 Translational Control

lated under these conditions. Such mRNAs include mammalian *ATF4*, *ATF5*, and yeast *GCN4* mRNAs (6, 16–20). Translation of *ATF4*, a member of the bZIP family of transcription factors responsible for inducing transcription of downstream stress-inducible genes, is governed by two uORFs within its 5'UTR (17, 20). Following translation of the upstream uORF, under basal conditions, ribosomes resume scanning and reinitiate translation at the downstream uORF, which leads to ribosome disassembly and prevents *ATF4* translation. In contrast, under conditions where eIF2 α is phosphorylated, reinitiating ribosomes have a higher probability of recruiting another eIF2 ternary complex downstream after the AUG codon of the downstream uORF and thereby initiate translation at the *ATF4* ORF. This mechanism is reminiscent of the classic model of reinitiation exemplified by the translational regulation of the yeast *GCN4* transcription factor (21). The expression of *ATF5*, another bZIP family member, is also regulated at the translational level through a similar mechanism involving uORFs in its 5'UTR, indicating that such a mechanism is conserved and likely important for regulating many mRNAs (18, 19).

The accumulation of unfolded proteins in the ER activates a multitude of intracellular signaling pathways, collectively referred to as the unfolded protein response (UPR) (22). One arm of the UPR switches on the ER-resident unfolded protein sensor, PERK, to repress global protein synthesis via eIF2 α phosphorylation, which in turn induces *ATF4* translation (16). *ATF4* activates the transcription of downstream stress-induced genes, including *CHOP* and *GADD34* (23). *GADD34* interacts with protein phosphatase 1 to dephosphorylate eIF2 α , which relieves the inhibition of translation (24, 25). This negative feedback loop is critical for translation of stress-induced genes and cell adaptation to ER stress (24–27). Although it is clear that *GADD34* transcriptional induction is important, the mechanism by which the *GADD34* transcript is translated during eIF2 α phosphorylation remains obscure.

In this study, we demonstrate that translation of the human and mouse *GADD34* mRNAs are governed through an uORF within its 5'UTR, which is responsible for translational repression during unstressed conditions and directs translation of *GADD34* during eIF2 α phosphorylation. Our results suggest that the human and mouse 5'UTRs use a distinct mechanism to ensure *GADD34* expression during cellular stresses that induce eIF2 α phosphorylation.

EXPERIMENTAL PROCEDURES

5' RACE and Plasmid Construction—The human 5'UTR *GADD34*-YFP, mouse 5'UTR *GADD34*-YFP, and human 5'UTR *ATF4*-YFP reporter plasmids were engineered using a two-part ligation strategy in the pcDNA3 vector. The human *GADD34* 5'UTR cDNA was synthesized by 5' RACE from total HepG2 RNA using the FirstChoice RLM-RACE system (Ambion) and PCR-amplified using a gene-specific primer, PrEJ18, and the 5' RACE-nested inner primer. The PCR product was TA-cloned (Invitrogen) and sequence-verified. The human *GADD34* 5'UTR fragment was digested with EcoRI and NcoI. The mouse *GADD34* 5'UTR cDNA was PCR-amplified using primers, PrEJ481 and PrEJ482, which contain a 5' EcoRI and a 3' NcoI site. The human *ATF4* 5'UTR was PCR-amplified

using primers, PrEJ279 and PrEJ420RR, and subsequently TA-cloned. *ATF4* 5'UTR was digested with a 5' EcoRI and a 3' BbsI site, which contains complementary ends with NcoI. The reporter enhanced YFP, eYFP (Clontech), was PCR-amplified using primers containing a 5' NcoI and a 3' XbaI site. The digested 5'UTR and amplified eYFP were cloned into EcoRI and XbaI sites of pcDNA3, resulting in fusion of the 5'UTR with YFP via an NcoI site. The NcoI site contains the AUG codon of eYFP. These ligations yielded the human *GADD34* 5'UTR-YFP (hGADD34-YFP), the mouse *GADD34* 5'UTR-YFP (mGADD34-YFP), and the human *ATF4*-YFP (hATF4-YFP) constructs. All mutant 5'UTRs, including mutations that either knocked out the AUG codon of each uORF, inserted codons between the start codon and stop codon of the mouse uORFs, or fused each uORF with the YFP ORF, were mutated using the QuikChange site-directed mutagenesis kit (Stratagene). All mutations were sequenced and verified.

The sequence of the mGADD34 uORFs overlap is 5'...cgacAUGAacc...-3'. The AUG start codon of uORF2 overlaps with the UGA of uORF1. To create the 1 and 2 codon insertion mutants, a codon (underlined) was inserted to produce 5'...cgacAUGUUGAacc...-3' (*1cod*) and 5'...cgacAUGUUUGUUGAacc...-3' (*2cod*).

Cell Culture and Stable Cell Lines—Mouse Hepa (1–6C) cells were generously provided by Maria Hatzoglou (Case Western University, Cleveland). HepG2 and Hepa cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (v/v), 2 mM glutamine, and 100 units of penicillin/streptomycin. Plasmid transfections in HepG2 cells were performed using Lipofectamine (Invitrogen) at 60% confluency. Stable cell lines were selected using Geneticin, and cells were passaged as pooled cell lines. Cells were treated either with 1 μ M thapsigargin (Sigma), 2 mM DTT, or 100 μ g/ml arsenite (Riedel de Haen) to activate eIF2 α kinases and induce eIF2 α phosphorylation.

Western Blot Analysis—Cells were washed two times with phosphate-buffered saline 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 and centrifuged to clear cell debris and nuclei. Protein concentration was determined using Bradford reagent (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride Immobilon-P or Immobilon-FL membrane (Millipore). Total eIF2 α was detected using a polyclonal antibody to the C terminus of eIF2 α (Cell Signaling, #9722), and phosphorylated eIF2 α was detected with an epitope-specific antiserum (Cell Signaling, #9721). Immunoblots were probed with *GADD34* (Santa Cruz Biotechnology, H-193), *CHOP* (Affinity Bioreagents MAI-250), *ATF4* (Santa Cruz Biotechnology, SC-200), and green fluorescent protein (Roche Applied Science) antibodies. The anti-green fluorescent protein antibody cross-reacts with the YFP reporter protein.

Sucrose Gradient Centrifugation and Polysome Analysis—Sucrose gradient centrifugation and polysome analysis was prepared as described (28). Briefly, prior to drug treatment and cell lysis, fresh media was added to cells 4–6 h earlier. Cells were

incubated with 100 $\mu\text{g/ml}$ cycloheximide for 3 min at 37 °C, washed three times with $1\times$ phosphate-buffered saline, and harvested directly on the plate using lysis buffer (15 mM Tris-HCl, pH 7.4, 15 mM MgCl_2 , 200 mM NaCl, 1% Triton X-100 (v/v), 100 $\mu\text{g/ml}$ cycloheximide, 1 mg/ml heparin). Nuclei and cell debris were cleared by centrifugation at $12,000\times g$ for 10 min at 4 °C, and the resulting supernatant was loaded onto a 10–50% (w/v) sucrose gradient composed of the lysis buffer minus the Triton X-100. The gradient was centrifuged at 35,000 rpm for 3 h in an SW41 rotor at 4 °C. Fractions were collected from the top using an ISCO fraction collector and a Brandel syringe pump system. To collect RNA, 3 ml of 8 M guanidine-HCl and 5 ml of EtOH were added to each fraction, precipitated, and resuspended in water. Equal volumes of fractionated RNA were subjected to Northern blot analysis.

Radiolabel Incorporation and Immunoprecipitation of Newly Synthesized Proteins—To examine newly synthesized proteins, cells were grown in Dulbecco's modified Eagle's medium minus methionine and cysteine supplemented with 10% (v/v) dialyzed fetal bovine serum for 25 min and metabolically labeled with 100 $\mu\text{Ci/ml}$ [^{35}S]methionine-cysteine for 20 min. To induce stress, 1 μM thapsigargin, 2 mM DTT, or 100 $\mu\text{g/ml}$ arsenite were incubated at the same time when cells were grown in Dulbecco's modified Eagle's medium minus methionine and cysteine. Radiolabeled cells were washed two times with $1\times$ phosphate-buffered saline and lysed in buffer containing 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. Equal amounts of proteins, determined by Bradford reagent, were incubated with anti-green fluorescent protein antibody (Roche Applied Science) and agarose-protein G beads according to the protocol recommended by Roche Applied Science. Proteins attached to the agarose-beads were recovered by boiling in Laemmli sample buffer and separated by SDS-PAGE. The gel was dried, subjected to PhosphorImager analysis, and quantified by ImageQuant (Storm, Amersham Biosciences).

Northern Blot Analysis—Total RNA (TRIzol, Invitrogen) or RNA purified from sucrose gradient centrifugation fractions were separated on a denaturing agarose gel and transferred to Zeta-probe membrane (Bio-Rad). Radiolabeled DNA hybridization probes were generated using the Radprime kit (Invitrogen). The amount of radiolabeled probe was quantitated by PhosphorImager analysis (Storm, Amersham Biosciences).

Oligonucleotide Sequences—The sequences were PrEJ18 (5'-CTACCCATGGGTCTGGGCGGCTGGGGGC-3'), PrEJ279 (5'-TTTCTACTTTGCCCGCCACAG-3'), PrEJ420 (RR-5'-CTAGGAAGACCCCATGGTTTCTTCAGCCCC-3'), PrEJ481 (5'-CTAGGAATTCGCTCTGAGTTTGTGGAAGATT-3'), and PrEJ482 (5'-CTAGCCATGGGTCTGGGCGGCGGGCTGCAC-3').

RESULTS

Translational Control during ER Stress in Liver Cells—To investigate the mechanisms of translational control during ER stress, human hepatoma HepG2 cells and mouse hepatoma Hepa cells were treated with UPR-inducing agents, thapsigargin or DTT. These cells were chosen because they are highly secretory and are sensitive to drugs that accumulate unfolded

proteins in the ER. Protein synthesis was measured by pulsed labeling with [^{35}S]methionine for the indicated times followed by quantitation of the total radioactive incorporation into newly synthesized polypeptides. Both thapsigargin and DTT treatment inhibited general protein synthesis as early as 15 min after incubation and was sustained for 30 min (Fig. 1, *A* and *B*). Notably, inhibition correlated with increased phosphorylation of eIF2 α observed at both time points (Fig. 1, *A* and *B*). A partial recovery in protein synthesis was observed upon prolonged incubation with thapsigargin or DTT (60 min), which correlated with reduced phosphorylation of eIF2 α (Fig. 1*A*). Incubation with thapsigargin or DTT for extended periods also elicited an increase in GADD34 protein levels, consistent with the fact that increased GADD34 protein levels mediate eIF2 α dephosphorylation (Fig. 2*C*) (26).

To examine the translation of specific and total RNAs during ER stress, lysates of cells untreated or treated with thapsigargin were separated by sucrose gradient centrifugation. In untreated cells, the majority of ribosomes sedimented to heavier molecular weight polysomes, indicating that most ribosomes are actively engaged in translation (Fig. 1*C*, *left panel*). In contrast, treatment of cells with thapsigargin resulted in dissociation of ribosomes from mRNAs and an increase in free 40 S and 60 S subunits (Fig. 1*C*, *right panel*). The loss of polysomes is in agreement with the significant decrease in protein synthesis during thapsigargin treatment.

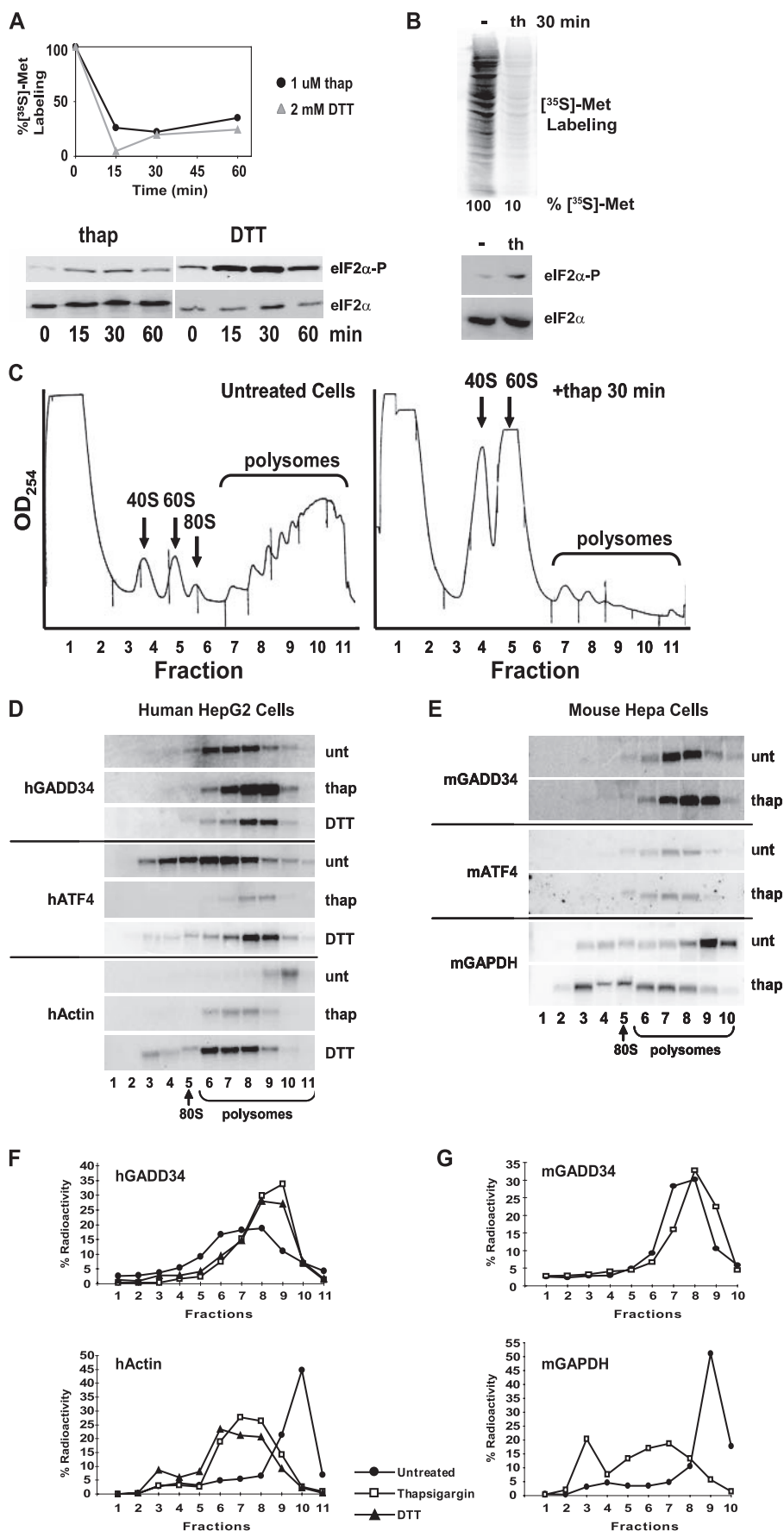
Polysome Association of GADD34 in Human and Mouse Liver Cells during ER Stress—We next asked whether GADD34 mRNAs are associated with polysomes during ER stress. To monitor the association of specific mRNAs with ribosomes, individual mRNAs within the sucrose gradient fractions were probed by Northern blot analysis. In untreated human HepG2 and mouse Hepa cells, actin and GAPDH mRNAs sedimented primarily to heavy molecular weight fractions 9 and 10, indicating that these mRNAs associated with many ribosomes and are actively translated (Fig. 1, *D–G*). Thapsigargin and DTT treatment resulted in a dramatic shift of actin and GAPDH mRNAs to lower molecular weight polysomes, consistent with these mRNAs being translationally repressed relative to those in untreated cells (Fig. 1, *D–G*). Conversely, ATF4 mRNA remained associated with polysomes during thapsigargin and DTT treatment in both cell lines as reported previously (Fig. 1, *D* and *E*) (16, 17). In HepG2 cells, ATF4 mRNA sedimented primarily with more ribosomes (*fractions 7–9*) under ER stress than under untreated conditions (*fractions 6–8*), indicating that this mRNA is translationally induced during eIF2 α phosphorylation (Fig. 1*D*). Similar to ATF4, human and mouse GADD34 mRNAs shifted to higher molecular weight fractions during thapsigargin and DTT treatments compared with those during unstressed conditions (Fig. 1, *D–G*). Therefore, like ATF4, both human and mouse GADD34 mRNAs possess mechanisms to direct translation during ER stress when eIF2 α is phosphorylated.

GADD34 Is Regulated at the Transcriptional and Translational Level during ER Stress—Given that the GADD34 mRNA was associated with more ribosomes (Fig. 1) and that GADD34 has been shown to be transcriptionally up-regulated during ER stress (24, 25), the increase in GADD34 protein expression is

5' UTR *GADD34* Translational Control

likely due to increases in both transcription and translation rates. To determine the contribution of translational control of *GADD34* during eIF2 α phosphorylation, we treated cells with the transcription inhibitor, actinomycin D, and thapsigargin. Incubation with actinomycin D inhibited the thapsigargin-induced transcription of *GADD34*, *ATF4*, and *CHOP*, consistent with previous reports that these genes are transcriptionally induced during ER stress (Fig. 2, A and B) (16, 24). Treatment of cells with thapsigargin alone or a combination of thapsigargin and actinomycin D induced a small but reproducible increase in *GADD34* protein levels, indicating that *GADD34* is translationally up-regulated (Fig. 2, C and D). Treatment with actinomycin D alone did not induce *GADD34* expression. Similarly, ATF4 protein expression was increased during thapsigargin and thapsigargin/actinomycin D treatments, whereas CHOP expression was only induced during thapsigargin treatment, in agreement that transcriptional induction is required for CHOP expression during ER stress (16). The induction of *GADD34* and ATF4 protein during thapsigargin/actinomycin D treatment was not as prominent as during thapsigargin treatment alone (Fig. 2, C and D), suggesting that transcriptional induction of *GADD34* and *ATF4* is required for maximal expression during eIF2 α phosphorylation.

The increase in *GADD34* protein during thapsigargin/actinomycin D treatment is likely due to the translational up-regulation of existing basal *GADD34* mRNAs. To confirm this, we analyzed the polysomal distribution of *GADD34* mRNAs in cells treated with thapsigargin alone or with thapsigargin/actinomycin D. Both treatments elicited a shift of *GADD34* mRNAs to higher molecular weight polysomal fractions as compared with that in untreated cells (Fig. 2E and supplemental Fig. S1). In contrast, GAPDH mRNAs shifted to lower molecular weight fractions during both treatments



(Fig. 2E and supplemental Fig. S1). Together with the Western blot analyses in Fig. 2C, these results demonstrated that *GADD34* mRNAs are translationally activated under thapsigargin-induced ER stress.

Interestingly, a 3-h thapsigargin treatment induced eIF2 α phosphorylation but by 6 h post-treatment, eIF2 α phosphorylation levels had decreased, likely due to maximal induction of *GADD34* protein (Fig. 2C). In contrast, during thapsigargin/actinomycin D incubation, eIF2 α remained phosphorylated at 6 h post-treatment, suggesting that the moderate induction of *GADD34* protein during this treatment was insufficient to reduce eIF2 α phosphorylation (Fig. 2C). In summary, the optimal induction of *GADD34* protein during eIF2 α phosphorylation is mediated through both transcriptional and translational controls of *GADD34*.

The Human and Mouse *GADD34* 5' UTRs Mediate Polysome Association during eIF2 α Phosphorylation—To begin elucidating the mechanism of *GADD34* translation, we first focused on its 5' UTR. An analysis of annotated mammalian *GADD34* mRNAs revealed two upstream open reading frames (uORFs) within the 5' UTR. The human (h*GADD34*), chimp and rat *GADD34* 5' UTRs contain two non-overlapping uORFs separated by 7–30 nucleotides, whereas the two uORFs of mouse (m*GADD34*), hamster, and bovine 5' UTRs are overlapping and out of frame by a single nucleotide (Fig. 3). Specifically, the uORFs overlap within the stop codon of the upstream uORF (uORF1) and the start AUG codon of the downstream uORF (uORF2) (Fig. 3).

To determine whether the *GADD34* 5' UTR is sufficient to direct translation during eIF2 α phosphorylation, we designed reporter RNAs containing the human or mouse *GADD34* 5' UTR fused upstream of an enhanced yellow fluorescent protein (YFP) ORF (Fig. 4). Stable HepG2 cell lines were generated that express different 5' UTR-YFP reporter RNAs, which are transcribed by the mammalian cytomegalovirus promoter. We first tested whether the *GADD34* 5' UTR could direct polysome association of the reporter RNA during thapsigargin-induced ER stress. Cell lysates were subjected to sucrose gradient centrifugation, and then fractionated. Following fractionation, the polysomal distributions of endogenous *GADD34*, *ATF4*, actin, GAPDH, and reporter RNAs were determined by Northern blot analysis. Quantitations of the Northern blots are shown in supplemental Fig. S2. For all stable cell lines, the endogenous actin, GAPDH, *GADD34*, and *ATF4* mRNAs had similar polysomal distributions to those observed with the parental HepG2 cell line (compare Figs. 1 (D–G) and 4 and supplemental Fig. S2), indicating that these cell lines were responsive to ER stress.

Briefly, actin and GAPDH mRNAs shifted to lower molecular weight polysomal fractions during thapsigargin-induced stress, whereas *GADD34* and *ATF4* mRNAs associated with heavier polysomal fractions under the same conditions (Fig. 4 and supplemental Fig. S2). The reporter RNA containing the human or mouse *GADD34* 5' UTRs (h*GADD34*-YFP or m*GADD34*-YFP, respectively) shifted to higher molecular weight polysomes during thapsigargin treatment (Fig. 4 (A and C) and supplemental Fig. S2), which is similar to the polysome distribution of the endogenous *GADD34* mRNA. This suggested that the h*GADD34* and m*GADD34* 5' UTRs were sufficient to direct translation during eIF2 α phosphorylation. As predicted, a reporter RNA containing the human *ATF4* 5' UTR (h*ATF4*-YFP) also associated with more ribosomes during ER stress (Fig. 4D), whereas a minimal YFP reporter RNA associated with fewer ribosomes during thapsigargin treatment (data not shown). Thus, the 5' UTRs of h*GADD34*, m*GADD34*, and h*ATF4* can confer resistance to the effects of eIF2 α phosphorylation-dependent translational repression during ER stress.

To test whether the uORFs of the h*GADD34* 5' UTR are important for translational regulation, the AUG codons of the upstream (uORF1) and downstream (uORF2) uORFs of h*GADD34* 5' UTRs were mutated to AUU (1&2KO h*GADD34*-YFP) (Fig. 4B). In untreated cells, the 1&2KO h*GADD34*-YFP RNA associated with more ribosomes (fractions 7 and 8) as compared with the wild-type h*GADD34*-YFP reporter RNAs (fractions 6 and 7) (Fig. 4 (A and B) and supplemental Fig. S2), indicating that intact uORFs were important for keeping basal translation repressed. Under thapsigargin treatment, the mutant reporter RNA shifted to lower molecular weight polysomes similar to that observed of the endogenous actin mRNA (Fig. 4 (A and B) and supplemental Fig. S2), demonstrating that mutating the uORFs sensitized the RNA to the inhibitory effects of eIF2 α phosphorylation. Thus, the h*GADD34* 5' UTR uORFs are important for repressing basal translation and for regulating translation during eIF2 α phosphorylation.

The Downstream uORF of Human *GADD34* Regulates Translation during eIF2 α Phosphorylation—To confirm that the h*GADD34* 5' UTR can direct translation during ER stress, the incorporation of [³⁵S]methionine/cysteine into newly synthesized YFP was measured by pulse labeling followed by immunoprecipitation. We first compared the translation efficiency of wild-type and mutant 1&2KO h*GADD34*-YFP RNA by measuring the amount of radioactive incorporation into newly synthesized YFP normalized to the amount of reporter YFP RNA (Fig. 5B, translational efficiency = radiolabel incorporation into YFP protein divided by YFP/GAPDH RNA levels). Trans-

FIGURE 1. Polysomal association of *GADD34* mRNA during eIF2 α phosphorylation in human HepG2 and mouse Hepa cells. HepG2 cells (A) or Hepa (1–6C) cells (B) were treated with 1 μ M thapsigargin or 2 mM DTT for the indicated times and subjected to [³⁵S]methionine/cysteine pulse-labeling. Radiolabel incorporation into newly synthesized protein was measured by trichloroacetic acid precipitation and normalized to that in untreated cells (100%) (A) or by autoradiogram (B) of SDS-PAGE analysis. The phosphorylation status of eIF2 α and the total eIF2 α in cell lysates were monitored by Western blot analysis using a phospho-specific antibody to phosphorylated eIF2 α (top) and an antibody that recognizes the C-terminal region of eIF2 α (bottom), respectively. C, sedimentation profiles at absorbance 254 nm of HepG2 lysates untreated (left) or treated with 1 μ M thapsigargin for 30 min (right). Cell lysates were fractionated by a 10–50% (w/v) sucrose gradient centrifugation. The top to bottom of the gradient is represented from left to right, respectively. The sedimentation of the 40 S, 60 S, 80 S fractions and polysomes are indicated. D and E, polysomal Northern blot analysis of RNA in fractions from HepG2 (D) and Hepa (E) cell lysates after 1 μ M 30 min thapsigargin (thap), 2 mM 30 min DTT, or untreated (unt) treatments as indicated to the right. The distribution of mRNAs is indicated to the left by Northern blotting of polysomal RNA. Fractions from top to bottom of the gradient are represented from left to right, respectively. Fractions containing 80 S and polysomes are indicated at the bottom. Quantitation of the Northern blots in D and E are shown in F and G, respectively. The amount of radioactive probe specific to the indicated mRNA in each fraction is indicated as a percentage of total radioactivity in all fractions within each sucrose gradient (% radioactivity).

5' UTR GADD34 Translational Control

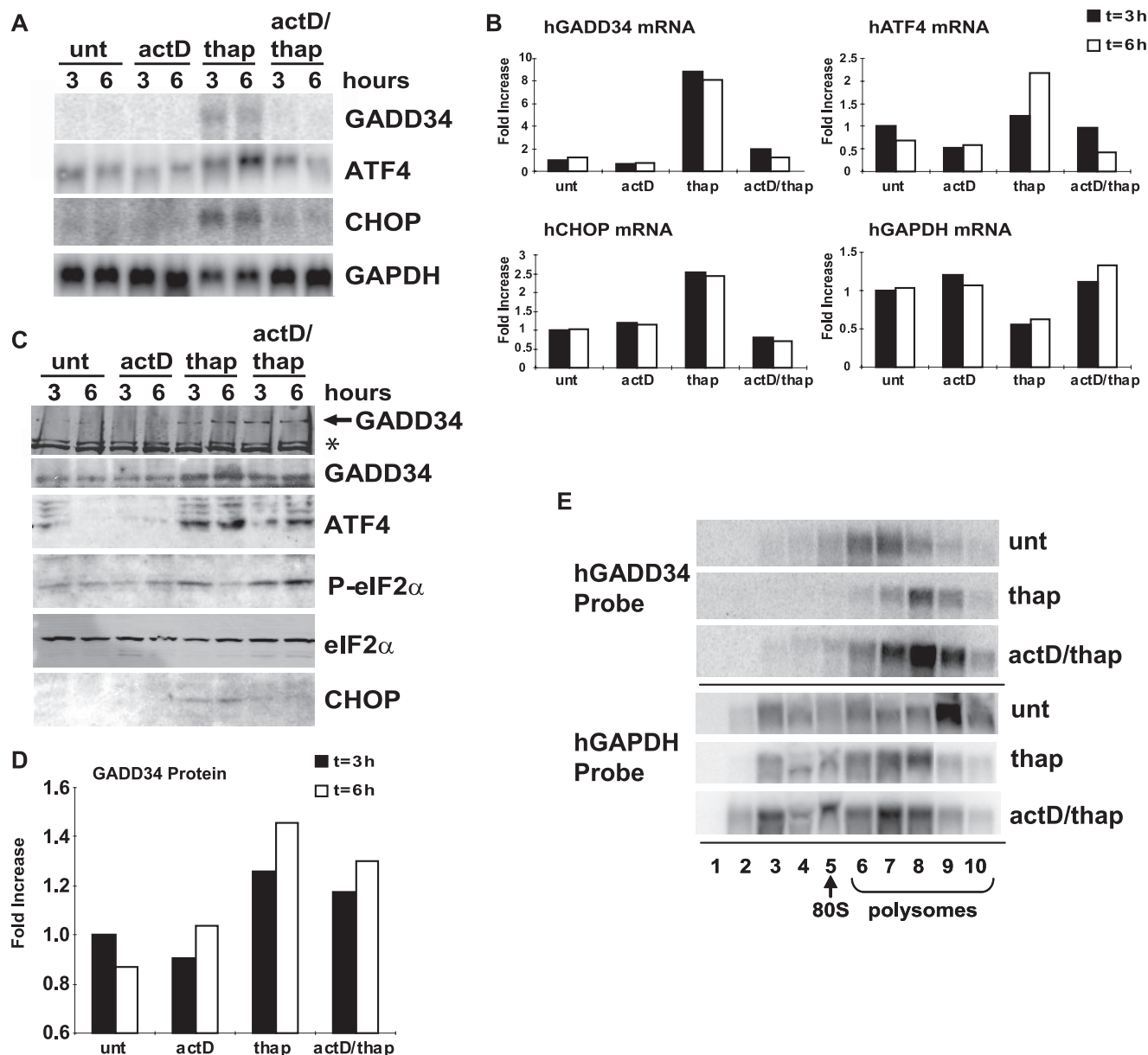


FIGURE 2. GADD34 expression in HepG2 cells during thapsigargin and actinomycin D treatment. *A*, Northern blots of RNA and (*C*) immunoblots of lysates from HepG2 cells alone (*unt*) or treated with 5 μ g/ml actinomycin D (*actD*), 1 μ M thapsigargin (*thap*), or thapsigargin/actinomycin D (*actD/thap*) for the indicated times. *A*, radiolabeled probes specific to *GADD34*, *ATF4*, *CHOP*, and *GAPDH* mRNAs (indicated to the right) were quantitated (*B*) using phosphorimaging analysis and normalized to the amount of RNA at the 3-h untreated time point. *C*, *GADD34*, *ATF4*, phosphorylated *eIF2 α* , total *eIF2*, and *CHOP* were detected by antibodies as indicated to the right. Representative Western blots of at least three independent experiments are shown. For *GADD34*, two independent experiments are shown, which shows reproducible induction of *GADD34* protein levels under thapsigargin alone and thapsigargin and actinomycin D treatment. The arrow indicates the migration of *GADD34* protein, and the asterisk indicates nonspecific proteins recognized by the *GADD34* antibody. *D*, quantitation of a representative *GADD34* immunoblot. The amount of *GADD34* protein was normalized to the amount of *GADD34* at the 3-h untreated time point (Odyssey-Licor). *E*, polysomal Northern blot analysis of RNA in fractions from untreated HepG2 cells (*unt*) or from cells after a 30-min treatment with 1 μ M thapsigargin (*thap*), 5 μ g/ml actinomycin D (*actD*), or 1 μ M thapsigargin (*actD/thap*) as indicated to the right. The distribution of *GADD34* and *GAPDH* mRNAs is shown by Northern blot analysis using radiolabeled DNA probes. Fractions from top to bottom of the gradient are represented from left to right, respectively. Locations of 80 S and polysomes across the gradient are indicated at the bottom. Quantitation of the amount of radioactivity within each fraction is shown in supplemental Fig. S1.

lation of 1&2KO hGADD34-YFP RNAs was \sim 29-fold higher than that of the wild-type hGADD34-YFP RNA, which is in agreement with the polysome distributions of the reporter RNAs (Fig. 4, *A* and *B*). Thus, the uORFs are important to repress basal translation during unstressed conditions.

Mutating hGADD34 uORF1 and uORF2 decreases the number of polysomes associated with the YFP mRNA in thapsigargin-treated cells (Fig. 4, *A* and *B*), suggesting that, in addition to

repressing translation during basal conditions, the *GADD34* 5' UTR is required for translation during stress conditions. To test whether the *GADD34* 5' UTR can direct translation during stress conditions, we measured the translation of YFP under the control of *GADD34* 5' UTR in the presence of thapsigargin or DTT, both of which induce ER stress and activate PERK. Treatment with either drug resulted in an overall inhibition of protein synthesis and induction of *eIF2 α* phosphorylation in all

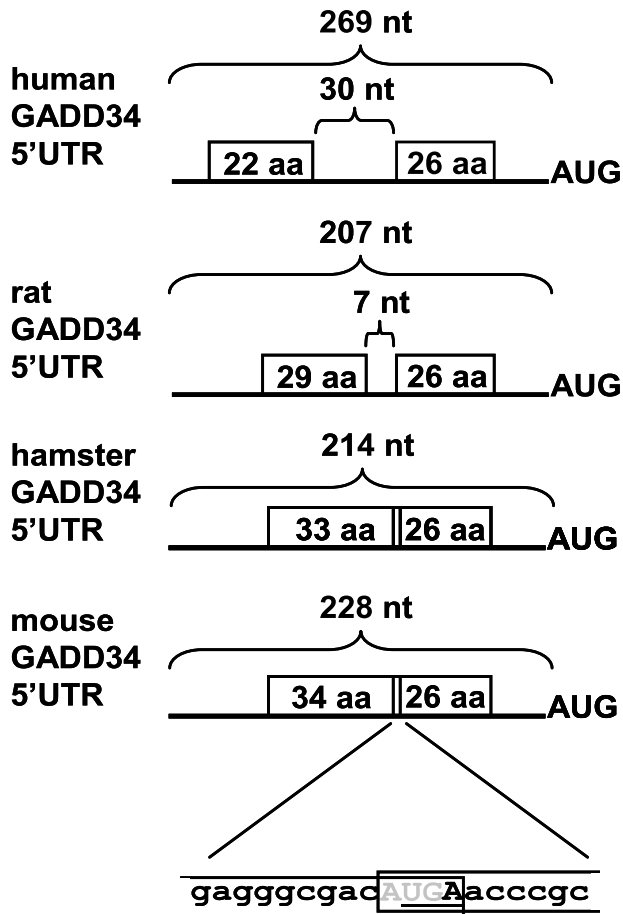


FIGURE 3. Schematic of uORFs within select mammalian GADD34 5'UTRs. Representative cDNAs encoding GADD34-related sequences in GenBank™ are shown, including human (NM_014330), rat (NM_133546), hamster (L28147), and mouse (NM_008654) GADD34 5'UTRs. The AUG codon of the GADD34 ORF is shown. The length of the 5' UTR and the space between uORFs are indicated above (nt, nucleotides). Each box represents uORF1 and uORF2 and the size of the predicted translated uORF (aa, amino acid length). A close-up view of the overlap of the mouse GADD34 uORFs is shown below. The upstream uORF (uORF1) overlaps with the downstream uORF (uORF2) by 1 nucleotide. The stop codon of uORF1 is underlined, and the AUG of uORF2 is in gray.

stable cell lines (Fig. 5A). Translation of YFP alone or mutant 1&2KO hGADD34-YFP was significantly inhibited by these drugs (Fig. 5, A and B). In contrast, translation of the wild-type hGADD34-YFP RNA was largely insensitive to the effects of eIF2 α phosphorylation as compared with 1&2KO hGADD34-YFP translation under the same treatments (Fig. 5, A and B). Arsenite, which induces oxidative stress and activates heme-regulated inhibitor, also leads to translation arrest. Like thapsigargin and DTT, arsenite inhibited the translation of YFP alone or mutant 1&2KO hGADD34-YFP but not that of wild-type hGADD34, indicating that the GADD34 5'UTR is required for translation during UPR as well as oxidative-stress response. As expected, the cricket paralysis virus intergenic region internal ribosome entry site (IGR IRES-YFP) and hATF4-YFP RNAs were also translated during eIF2 α phosphorylation (Fig. 5A). The cricket paralysis virus intergenic region IRES can recruit the ribosome independently of initiation factors and has been shown to be resistant to eIF2 α phosphorylation-dependent translational repression (29–31). Similarly, the mechanism via

the ATF4 5'UTR uORFs promotes translation during eIF2 α phosphorylation (17, 20). Levels of reporter RNAs were similar during eIF2 α phosphorylation, thus the differences in YFP expression were due to alterations in translational efficiencies (Fig. 5, A and B). Therefore, polysome association of the wild-type hGADD34-YFP RNA during ER stress reflected protein synthesis by ribosomes.

The two uORFs (uORF1 and uORF2) within the hGADD34 5'UTR are reminiscent of the non-overlapping uORFs found within the yeast *GCN4* and mouse *ATF4* 5'UTRs. But they differ in that hGADD34 uORFs are separated by a shorter (30 nucleotide) spacer. Previous reports have found that an intercistronic space of only 30 nucleotides limited the time for the scanning ribosomes to re-acquire another ternary complex before reaching the start codon of the downstream uORF, even during basal conditions where ternary complex is abundant (32–34). This argues against a model where reinitiation represses hGADD34 translation. However, it remains possible that the intergenic distance between the two uORFs within the hGADD34 5'UTR is not limiting, in which case reinitiation may account for the repression under basal conditions. To investigate this possibility that reinitiation takes place at the downstream uORF, we first determined whether the individual uORFs of hGADD34 5'UTR are inhibitory. To address this, we mutated the AUG codon of the upstream (uORF1) or downstream (uORF2) uORFs to AUU, separately. Mutating the uORF1 or uORF2 (1KO and 2KO, respectively) repressed basal translation to different extents. An intact uORF1 moderately inhibited translation (4.5-fold), whereas an intact uORF2 inhibited translation significantly (0.5-fold) as compared with the reporter RNA in which both uORFs are mutated (29.4 fold) (1&2KO hGADD34-YFP) (Fig. 5, A and B). This is in contrast to the upstream uORF of *ATF4*, which stimulates efficient reinitiation at the downstream AUG codon (17, 20). Although the arrangement of the hGADD34 uORFs is similar to that of *ATF4*, both hGADD34 uORFs were inhibitory thus suggesting that the mechanism of translational repression of hGADD34 is distinct from that of *ATF4* and may not involve reinitiation at the downstream uORF.

We next asked whether hGADD34 5'UTR lacking either uORF1 or uORF2 could still direct translation during thapsigargin treatment. Compared with basal translation, an intact uORF2 within the GADD34 5'UTR directed translation during thapsigargin-induced ER stress (Fig. 5, A and B). In contrast, translation of the mutant 2KO hGADD34-YFP was significantly repressed similar to the reporter RNA lacking both uORFs. In all cases, the reporter RNA levels were unaltered during this treatment (Fig. 5, A and B). Thus, the downstream hGADD34 uORF2 was inhibitory during unstressed conditions and directed translation of GADD34 during eIF2 α phosphorylation.

The Overlapping uORFs of the Mouse GADD34 5'UTR Control Translation during eIF2 α Phosphorylation—Unlike the human version, the mouse GADD34 5'UTR contains two overlapping uORFs (Fig. 3). To investigate whether the mGADD34 uORFs control translation, we engineered mutant mGADD34-YFP expression constructs in which the AUG codons of each or both uORFs were mutated. Because the AUG codon of the

5' UTR GADD34 Translational Control

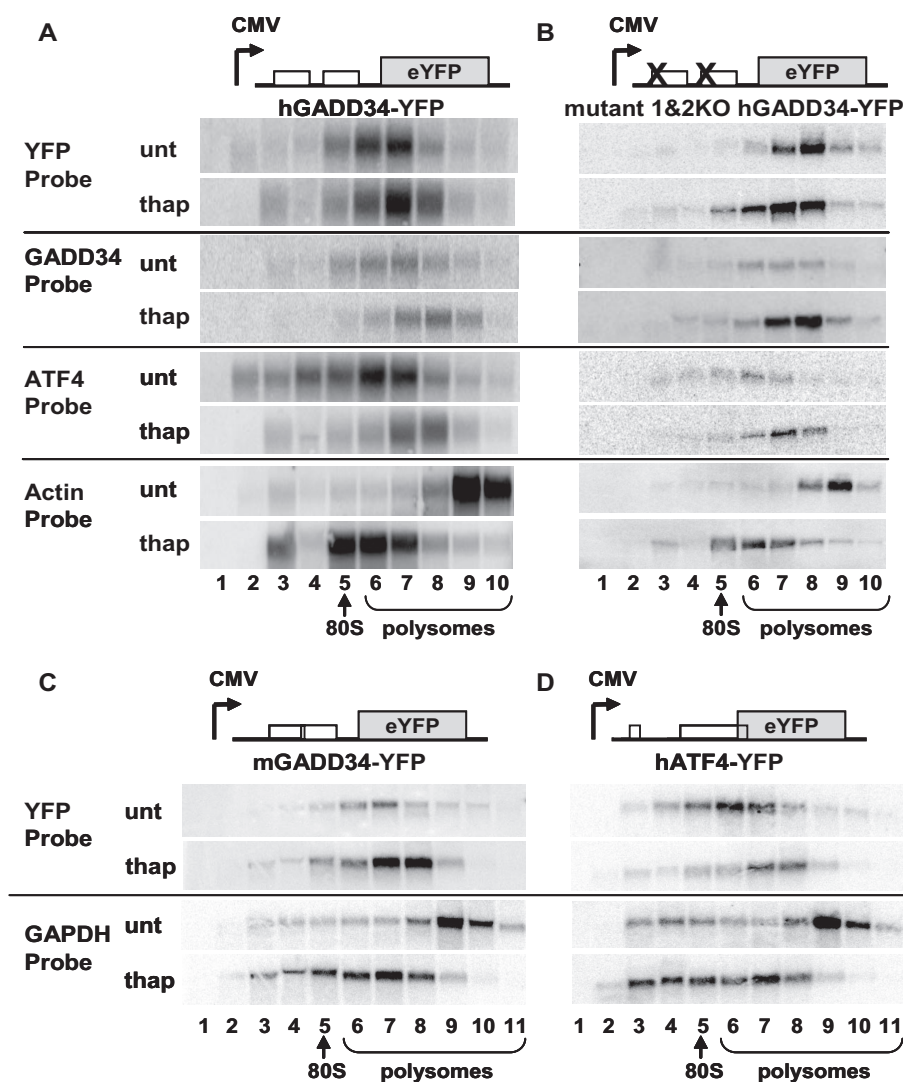


FIGURE 4. The 5' UTR of human and mouse *GADD34* mediates polysome association during thapsigargin treatment in HepG2 cells. Sucrose gradient density centrifugation and fractionation of lysates from stable cell lines expressing reporter YFP RNA fused with wild-type human *GADD34* 5' UTR (hGADD34-YFP) (A), mutant human *GADD34* 5' UTR (1&2KO hGADD34-YFP) (B), mouse *GADD34* 5' UTR (mGADD34-YFP) (C), or human *ATF4* 5' UTR (hATF4-YFP) (D) that were incubated in the absence (*unt*) or presence of 1 μ M thapsigargin for 30 min (*thap*) were performed. The reporter RNAs were transcribed by the cytomegalovirus promoter (CMV). The distribution of specific RNAs across the gradient was detected by Northern blot analysis as indicated to the left. Fractions from top to bottom of the gradient are represented from left to right, respectively. Fractions that contain the 80 S and polysomes are indicated below the fractions. Quantitations of the amount of radioactivity within each fraction are shown in supplemental Fig. S2.

downstream uORF2 overlaps with the UGA stop codon of the upstream uORF1, we engineered two mutations that mutate the AUG codon but restored the UGA stop codon. The first mutation altered the AUG codon to a UUG and the second to a CUG. We produced these two mutants to confirm that altering the AUG codon did not have an effect on the translation of the upstream uORF. In all experiments, both of these mutations produced similar results and will be referred to as 2KO mGADD34 5' UTR.

Stable HepG2 cell lines expressing the wild-type and mutant mGADD34-YFP RNAs were selected, and YFP expression was quantified by immunoprecipitation as described above. As compared with unstressed conditions, translation of wild-type mGADD34-YFP RNA was maintained during thapsigargin treatment, which is in agreement

with the polysome distribution of this reporter RNA (Figs. 4C, 6A, and 6B). Mutating both mGADD34 uORFs or just uORF2 (2KO) directed higher basal YFP expression, indicating that the mGADD34 uORF2 was inhibitory (Fig. 6, A and B). Thapsigargin treatment inhibited translation of 1&2 KO mGADD34-YFP and 2KO mGADD34-YFP RNAs similar to that observed of total protein synthesis in the cell (~20%) (Fig. 6B). In contrast, an intact uORF2 (1KO) did not significantly affect basal YFP expression and, like the wild-type version, the 1KO mGADD34-YFP RNA remained translated during thapsigargin treatment. For all stable cell lines, the level of reporter RNA was unaltered under basal and stressed conditions (Fig. 6, A and B). In summary, like the human hGADD34, the uORF2 of mGADD34 5' UTR was inhibitory under basal conditions and mediates translation during ER stress when eIF2 α was phosphorylated. To confirm that the mouse uORF2 was sufficient to direct translation during ER stress, we monitored the polysome distribution of 1KO mGADD34-YFP RNA in untreated and thapsigargin-treated cells. The reporter RNA shifted to heavier polysomes during ER stress, similar to the endogenous *GADD34* mRNA (data not shown).

In the case of the *ATF4* 5' UTR, ribosomes resume scanning after translation of the upstream uORF and re-acquire another ternary complex for reinitiation at the downstream AUG. The key feature

of this mechanism relies on sufficient spacing between the uORFs to give time for scanning ribosomes to re-acquire eIF2-GTP/Met-tRNA_i complex (32). In contrast, the overlap of the mGADD34 uORFs suggests that translational control via the mGADD34 5' UTR is distinct from the *ATF4* reinitiation mechanism. The UGA of the upstream uORF overlaps with the AUG of the downstream uORF in the -1 frame. To determine whether spacing between the UGA and AUG codon is important for regulation, we engineered mutant mGADD34 5' UTR-YFP reporters with either one or two extra codons inserted between the UGA of the upstream uORF and the AUG of the downstream uORF (see "Experimental Procedures" for specific details on the insertion sequence). Adding one or two extra codons maintained basal translation at a low level similar to the wild-type mGADD34-YFP RNAs (Fig. 6, A and B). Interest-

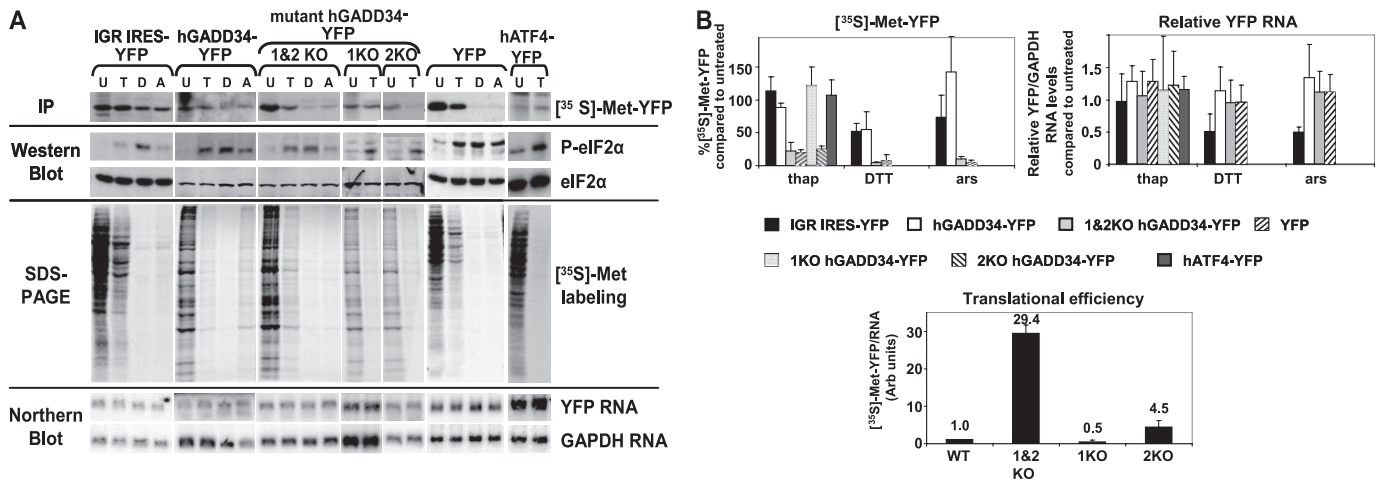


FIGURE 5. The uORFs of hGADD34 5' UTR mediate translation regulation during eIF2 α phosphorylation. In *A*: *Top row*, immunoprecipitates of [³⁵S]methionine-labeled YFP from lysates of cells expressing IGR IRES-YFP, hATF4-YFP, wild-type hGADD34-YFP, YFP alone, or mutant human GADD34 5' UTR fused with YFP where the AUG codon of the upstream uORF (1KO), downstream uORF (2KO), or both uORFs were mutated (1KO, 2KO, and 1&2KO, respectively) were either left untreated (U) or treated with 1 μ M thapsigargin (T), 2 mM DTT (D), or 100 μ g/ml arsenite for 45 min (A). Cells were pulse-labeled with [³⁵S]methionine for 20 min prior to harvesting. Immunoprecipitates were separated by SDS-PAGE and exposed by phosphorimaging analysis. *Second row*, immunoblots of lysates using antibodies that recognize phospho-eIF2 α or total eIF2 α . *Third row*, lysates were subjected to SDS-PAGE analysis and exposed to autoradiography. *Bottom row*, in parallel, RNA from treated cells was subjected to Northern blot analysis using probes specific for YFP or GAPDH. *B*, quantitation of newly synthesized [³⁵S]methionine-labeled YFP immunoprecipitates (*top left*) and levels of YFP RNA normalized to GAPDH (*top right*) as described in *A*. Each bar represents the percent of [³⁵S]methionine-labeled YFP expressed or the YFP RNA levels during the indicated drug treatments as compared with that in untreated cells, which is set at 100%. For Northern blot analysis, YFP mRNA levels were normalized to GAPDH mRNA levels. *Bottom*, translational efficiency of reporter YFP RNA in unstressed cells was calculated by the amount of newly synthesized [³⁵S]methionine YFP protein normalized to YFP/GAPDH mRNA. *Above each bar* shows the average translational efficiency normalized to the wild-type hGADD34-YFP RNA as 1. Shown are averages \pm S.D. from at least three independent experiments.

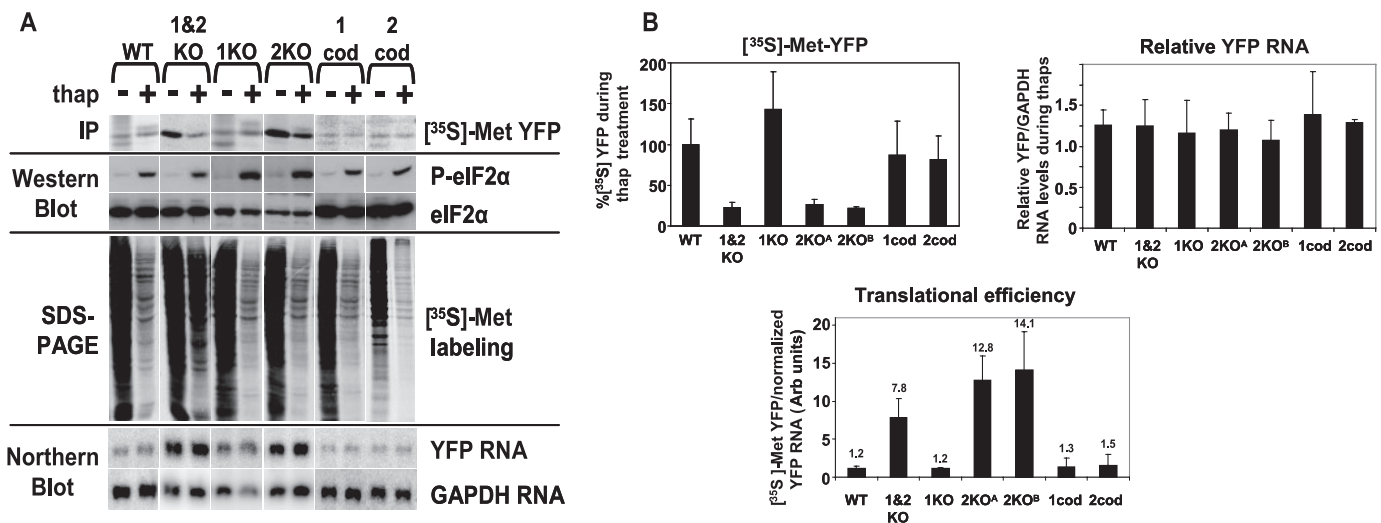


FIGURE 6. The overlapping uORFs of the mouse 5' UTR mGADD34 mediate translational control during eIF2 α phosphorylation. *Top row*, immunoprecipitates of [³⁵S]methionine-labeled YFP from lysates of cells stably expressing wild-type or mutant mouse 5' UTR mGADD34-YFP reporter RNAs as indicated above under untreated or 1 μ M thapsigargin treatment for 45 min. 1&2KO, 1KO, and 2KO represent mutant mouse mGADD34 5' UTR reporter RNAs in which the AUG start codon of uORF1 (1KO), uORF2 (2KO), or both (1&2KO) uORFs was mutated. Mutations that inserted one (1cod) or two (2cod) codons between the AUG codon of the uORF2 and the stop codon of the upstream uORF1 were engineered into the 5' UTR mGADD34-YFP reporter RNA (see "Experimental Procedures" for sequence). *Second row*, immunoblots of lysates using antibodies that recognize phospho-eIF2 α or total eIF2 α . *Third row*, cells were pulse-labeled with [³⁵S]methionine for 20 min prior to harvesting. Lysates were subjected to SDS-PAGE analysis and exposed to autoradiograph. *Bottom row*, in parallel, RNA from treated cells was subjected to Northern blot analysis using probes specific for YFP or GAPDH. *B*, quantitation of [³⁵S]methionine-labeled YFP immunoprecipitates (*top left*) and of YFP RNA levels normalized to GAPDH (*top right*). *Bottom*, each bar represents the percentage of [³⁵S]methionine-labeled YFP expressed or YFP RNA levels during the thapsigargin treatment as compared with that in untreated cells. Translational efficiency of reporter YFP RNA in unstressed cells was calculated by the amount of newly synthesized [³⁵S]methionine precipitates normalized to YFP/GAPDH mRNA levels. *Above each bar* shows the average translational efficiency normalized to the wild-type mGADD34-YFP RNA. Shown are averages \pm S.D. from at least three independent experiments.

ingly, the reporter RNAs containing the extra codons were still translated during thapsigargin treatment, indicating that increasing the overlap of uORFs by two codons does not significantly affect translational control mediated by the mGADD34 5' UTR during eIF2 α phosphorylation (Fig. 6, *A* and *B*).

Although translation is relatively resistant to eIF2 α phosphorylation, the hGADD34-YFP, mGADD34-YFP, and hATF4-YFP RNAs do not incorporate more radiolabel during ER stress when compared with basal conditions, which is contrary to its polysome distribution where these reporter RNAs shifted to

5' UTR GADD34 Translational Control

higher molecular weight polysomes during thapsigargin treatment (compare Figs. 5 and 6 with Fig. 4, A, C, and D). From the polysome distribution, we would predict that translation of these reporter RNAs would be increased during stress. This may reflect differences in protocols between the two experiments. In the immunoprecipitation experiments, cells were amino acid-starved prior to labeling, whereas the cells were not in the polysome analysis protocol. To test this hypothesis, we analyzed the polysomal distribution of mGADD34-YFP in cells that were amino acid-starved. Unlike the shift to higher molecular weight polysomes during thapsigargin treatment (Fig. 4C and supplemental Fig. S2), the mGADD34-YFP RNAs did not shift in cells that were starved and treated with thapsigargin (supplemental Fig. S3). Similarly, the endogenous *GADD34* mRNA also did not shift to higher molecular weight polysomes during starvation/thapsigargin treatment (compare Fig. 1F and supplemental Fig. S3). Therefore, amino acid starvation inhibited the 5'UTR-dependent translational activation of *GADD34* during ER stress. The inhibitory effect of amino acid starvation on thapsigargin-induced translation of *GADD34* remains to be investigated.

Leaky Scanning Permits Translation of uORF2 within the hGADD34 and mGADD34 5'UTRs—The results indicate that the 5'UTR uORF2 represses translation of hGADD34 and mGADD34 under unstressed conditions. Mutating uORF2 led to moderately higher basal levels of translation, suggesting that uORF1 does not inhibit scanning ribosomes. However, our data do not formally exclude that ribosome reinitiate after translation of uORF1, a scenario similar to the *ATF4* 5'UTR. Alternatively, ribosomes may skip the first AUG codon and continue scanning, an event called leaky scanning (35). The major determinants for AUG recognition by scanning ribosomes are the nucleotides surrounding the AUG codon called the Kozak consensus (13, 36, 37). In general, an optimal Kozak consensus contains both an A/G at -3 and a G at $+4$ given that A of AUG is $+1$. An AUG codon that contains either of these determinants is considered to be in strong AUG context, whereas an AUG lacking both determinants is considered a weak context. The AUG codons for both uORFs in the hGADD34 and mGADD34 5'UTRs are in poor context, thus predicting that leaky scanning occurs within the 5'UTR.

To determine whether the uORFs are recognized by scanning ribosomes, we engineered mutant hGADD34-YFP and mGADD34-YFP reporter RNAs in which the stop codon for each uORF was mutated and the uORF fused in-frame with the reporter YFP ORF (Fig. 7). The design of hGADD34 uORF1-YFP was such that the AUG codon of uORF2 is in the same frame, thus we can determine whether scanning ribosomes skip the first AUG codon. For the mGADD34 uORF1-YFP, the AUG codon of uORF2 was mutated to fuse uORF1 in frame with the YFP ORF.

Under basal conditions, translation of uORF2-YFP was readily observed from the human and mouse versions of *GADD34* 5'UTR constructs (Fig. 7). Thapsigargin treatment moderately inhibited uORF2-YFP translation. In contrast, we observed different results with uORF1-YFP. The hGADD34 uORF1-YFP RNA produced two protein products representing translation from the AUG codon of both uORF1 and uORF2

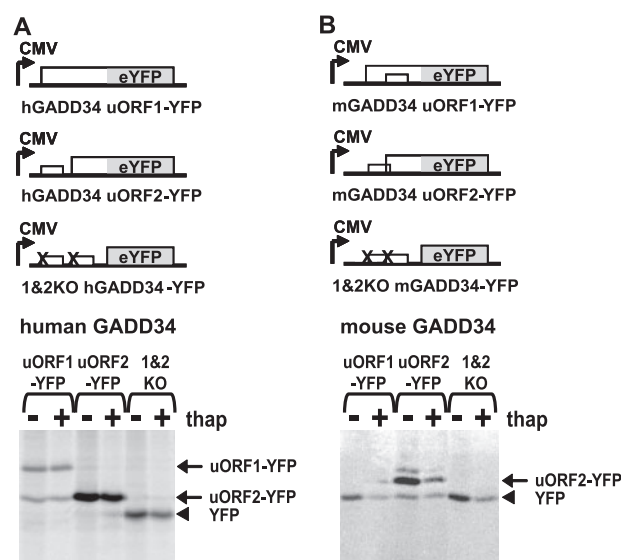


FIGURE 7. The downstream uORF2 of hGADD34 and mGADD34 5'UTRs are translated efficiently in HepG2 cells. Schematics of human (A) or mouse (B) *GADD34* 5'UTR-YFP reporter RNAs stably expressed in HepG2 cells. The upstream (uORF1) or downstream (uORF2) uORFs were engineered such that the reading frames of the uORF were fused with the reporter YFP ORF. For the human hGADD34 uORF1-YFP RNA, the uORF2 is in-frame with uORF1 and the YFP ORF, thus both uORF1-YFP and uORF2-YFP fusion protein products can be monitored. Below, immunoprecipitates of newly synthesized ^{35}S -labeled YFP protein from lysates of cells in the presence or absence of $1\ \mu\text{M}$ thapsigargin. Reactions were resolved on an SDS-PAGE and exposed by phosphorimaging analysis. uORF1-YFP, uORF2-YFP, and YFP ORF proteins are indicated to the right.

(Fig. 7A). Thus, a proportion of scanning ribosomes skips the first AUG codon and directs translation from the downstream uORF2 AUG codon, indicating that leaky scanning was involved. Interestingly, thapsigargin treatment did not significantly affect translation of hGADD34 uORF1-YFP or uORF2-YFP or the extent of leaky scanning. Surprisingly, the mGADD34 uORF1-YFP did not produce the expected fusion uORF1-YFP protein and only resulted in translation of the YFP ORF (which is fused in-frame) (Fig. 7B). Therefore, scanning ribosomes most likely skip uORF1 to initiate translation at the downstream YFP ORF AUG codon. Levels of reporter uORF1-YFP and uORF2-YFP RNA were similar (data not shown). In summary, the 5'UTRs of human and mouse *GADD34* permitted a significant amount of leaky scanning of ribosomes to bypass the AUG codon of uORF1 and directed translation of uORF2, which is a strong barrier of scanning ribosomes.

DISCUSSION

eIF2 α phosphorylation elicits a cascade of signaling events that reprograms gene expression in order for the cell to adapt to environmental stress. Paradoxically, translational inhibition via eIF2 α phosphorylation activates the translation of a subset of mRNAs (e.g. *ATF4* and yeast *GCN4*), which encode transcription factors that activate downstream stress-response genes. A key component of this cellular response is the induction of *GADD34* protein expression. In this report, we demonstrate that *GADD34* induction is in part mediated through a 5'UTR mechanism that increases translation during ER stress. *GADD34* mRNAs associate with and shift to heavier polysomes during thapsigargin- and DTT-induced ER stress (Fig. 1, D–G),

indicating that *GADD34* is translated under conditions of general translational arrest when eIF2 α is phosphorylated. Specifically, the 5'UTR of both human and mouse *GADD34* mRNAs is essential and sufficient to direct efficient translation during ER stress. Through reporter constructs, we showed that the *GADD34* 5'UTR induced re-distribution of reporter mRNAs to heavier polysomes during eIF2 α phosphorylation, which is similar to the distribution of endogenous *GADD34* mRNAs (Fig. 4 and supplemental Fig. S2). Our data, together with earlier reports (38), support the notion that induction of *GADD34* translation during eIF2 α phosphorylation plays an important role in the general stress response, which alleviates cellular stress, including ER stress, oxidative stress, and hypoxia.

GADD34 is an essential component of the UPR (24, 26). *GADD34* interacts with protein phosphatase 1 via its C-terminal region to dephosphorylate eIF2 α , leading to translational recovery (24, 26, 39, 40). Ectopic expression of a truncated *GADD34* protein lacking the catalytic C-terminal region in mouse embryo fibroblasts prevents dephosphorylation eIF2 α and blocks translational recovery during ER stress, resulting in premature apoptosis (24). The heightened sensitization to cell death is likely due to translational repression of stress-induced transcripts, such as the chaperone BiP, which are normally expressed during ER stress (26). Thus, the increased expression of *GADD34* results in a negative feedback loop to enhance the translation of stress-induced mRNAs during eIF2 α phosphorylation, which are essential for cellular survival and adaptation to environmental stress. *GADD34* is also regulated at the transcriptional level, which is induced in part by ATF4 and CHOP, which are expressed during ER stress (25, 27). However, it was unclear whether the increase in *GADD34* mRNA alone yielded sufficient *GADD34* protein during eIF2 α phosphorylation to promote the negative feedback loop. Our data demonstrates that *GADD34* is translationally induced to ensure maximal expression during ER stress. We showed that *GADD34* mRNAs associated with more ribosomes during ER stress, suggesting that eIF2 α phosphorylation leads to increased *GADD34* translation (Fig. 1, D–G). In addition, *GADD34* protein increased under thapsigargin treatment in the presence of the transcription inhibitor actinomycin D (Fig. 2, C and D). This indicated that ongoing transcription was not required for *GADD34* induction and that existing basal *GADD34* mRNAs can be translated during ER stress. In support of this, we showed that *GADD34* mRNAs associated with higher molecular weight polysomes in cells treated with thapsigargin and actinomycin D (Fig. 2E and supplemental Fig. S2). However, the induction of *GADD34* protein was moderate in thapsigargin/actinomycin D-treated cells compared with that of cells treated with thapsigargin alone and was not sufficient to reduce eIF2 α phosphorylation at later time points (Fig. 2D). These results are in agreement with previous observations that impaired expression of *GADD34* in CHOP^{-/-} mice embryonic fibroblasts leads to persistent eIF2 α phosphorylation and loss of protein synthesis recovery (27). Therefore, the coordinated transcriptional and translational controls of *GADD34* are necessary for optimal expression to dephosphorylate eIF2 α and for translational recovery.

Our results revealed that the uORFs of the human and mouse *GADD34* 5'UTR played a significant role under basal and stress conditions. 5'UTR uORFs have been shown previously to be important for translational control of several mRNAs (41). In general, uORFs act as barriers to scanning ribosomes thereby modulating translation of the authentic ORF. Under certain cellular conditions, ribosomes can bypass the uORFs and reinitiate translation at the main ORF. The best studied mechanism of translational reinitiation is the one governing *ATF4*, *ATF5*, and yeast *GCN4* translation. The major premise of this mechanism is that the translation of the upstream uORF stimulates translation or reinitiation at a downstream AUG, whereas translation of the downstream uORF leads to translation termination and dissociation of ribosomes. Following translation of the upstream uORF, if eIF2 levels become limiting (*i.e.* eIF2 α is phosphorylated), the recruitment of the ternary complex by the ribosome is markedly reduced and as a result ribosomes have a higher probability of reinitiating translation after the downstream uORF and thereby reinitiate translation at the authentic ORF. Major determinants that control the extent of reinitiation are the intercistronic space between the uORFs and the length of the upstream uORF. After translation of uORF1, the probability of reinitiating at the downstream uORF2 depends on the length of the intercistronic space to allow for scanning ribosomes to re-acquire the ternary complex (32–34). The shorter the intercistronic space, the greater chance that scanning ribosomes will bypass uORF2. Another feature of this mechanism is that the extent of reinitiation decreases the longer the uORF, whereas efficient reinitiation occurs with translation of very short uORFs (2–3 amino acids long) (21, 42). *ATF4*, *ATF5*, and *GCN4* all contain short uORFs. It has been proposed that ribosomes that translate a short uORF are still bound to certain initiation factors (*i.e.* eIF4G), which promotes reinitiation (43). However, when longer uORFs are translated, these factors fall off the ribosome, thus preventing reinitiation (43).

Our data indicate that the two uORFs within the human and mouse *GADD34* 5'UTRs control translation through a mechanism that is distinct from the well studied reinitiation mechanism. First, neither the human *GADD34* uORF1 nor the mouse *GADD34* 5'UTR can promote reinitiation (Fig. 5 and 6). In fact, the human uORF1 moderately inhibited scanning ribosomes as compared with a 5'UTR with no uORFs (Fig. 5). Secondly, human and mouse uORF1s are poorly translated and a significant proportion of ribosomes scan past uORF1 to initiate translation at a downstream AUG (Fig. 7). In contrast, the uORF1 of *ATF4* is readily translated, which is a prerequisite for reinitiation at the uORF2 under basal conditions and at the main *ATF4* ORF during eIF2 α phosphorylation (17, 20). Instead, the presence of the *GADD34* uORF2 was sufficient for translational control. An intact uORF2 reduces translation during unstressed conditions and is important for translational induction during stressed conditions, which incidentally are all properties that are observed with the endogenous *GADD34* mRNA (Fig. 4–6, data not shown).

In addition, the features and arrangement of the *GADD34* uORFs do not fit the model of reinitiation. The mouse *GADD34* uORFs overlap by one nucleotide, whereas the human uORFs are separated by 30 nucleotides. In the case of the mouse

5' UTR GADD34 Translational Control

GADD34 uORFs, if reinitiation plays a role in this regulation, ribosomes would have to reinitiate backwards (following translation of uORF1) to start translation at the AUG codon of uORF2. Although there have been reports of backward reinitiation, the extent of this was either very inefficient or requires a specialized RNA sequence that binds to ribosome-recruiting factors (*i.e.* eIF3) (42, 44). When extra codons were introduced to extend the overlap between the start and stop codons, the mutant mouse *GADD34* 5'UTR was still functional like the wild-type version, arguing against backward reinitiation (Fig. 6). In the case of the human *GADD34*, the uORFs are separated by a 30-nucleotide intercistronic spacer, which is considerably shorter than those separating uORFs in human *ATF4* (87 nucleotides), *ATF5* (110 nucleotides), and yeast *GCN4* (198 nucleotides between uORF1 and uORF4) uORFs. Furthermore, whereas the upstream uORF of *ATF4*, *ATF5*, and *GCN4* 5'UTRs are short, uORF1 of the human *GADD34* is relatively long (22 amino acids), which is predicted to greatly decrease the efficiency of reinitiation (32–34). These features argue that leaky scanning occurs on the *GADD34* 5'UTR to bypass uORF1 and initiate translation at uORF2.

The uORF2 of *GADD34* is a strong barrier for scanning ribosomes, which efficiently represses *GADD34* translation under basal conditions. A probable scenario is that, after translating uORF2, ribosomes dissociate, thus inhibiting translation of the *GADD34* ORF. Alternatively, the uORF2 may mediate other effects. It has been previously shown that the uORF in fungal mRNAs that encode a subunit of Arg-specific carbamoyl phosphate synthetase controls translation by stalling scanning ribosomes and mediating nonsense-mediated decay (45, 46). In this case, the translated coding sequence of the fungal uORF dictates translational control (47, 48). Interestingly, a comparison of mammalian *GADD34* 5'UTRs reveal that uORF2 is highly conserved. The human, chimp, and rat *GADD34* 5'UTRs contain non-overlapping uORFs, and the mouse and hamster versions contain overlapping uORFs. Although the uORF1s differ in length and are poorly conserved, the uORF2s are exactly 26 amino acids long and contain 18/26 identical amino acids. It remains to be determined whether the amino acid or nucleotide sequence composition within uORF2 plays a significant role in translational control during eIF2 α phosphorylation. The high degree of conservation of uORF2 further strengthens the idea that translational control is mediated by this uORF specifically. However, it is unclear why a dispensable uORF1 has evolved within the *GADD34* 5'UTR. It is possible that uORF1 has an unexplored role under a different set of cellular stress conditions.

An unresolved question is how do scanning ribosomes bypass uORF2 and reach the *GADD34* ORF when eIF2 α is phosphorylated? Other than the reinitiation mechanism, additional mechanisms that can bypass eIF2 α phosphorylation have been described. One such mechanism proposes that ribosomes are directly recruited to the 5'UTR downstream of uORF2, possibly through an IRES. The most unique example is via the cricket paralysis virus intergenic region IRES, which can bypass the requirement for all initiation factors to recruit the ribosome and can induce translation during eIF2 α phosphorylation (31,

49). Another example is *cat-1*. In response to amino acid starvation, translation of *cat-1* is induced, which is mediated in part by an IRES within its 5'UTR and requires translation of an uORF (50). However, our data indicate that an IRES-like mechanism does not direct *GADD34* translation.³

Interestingly, an inhibitory uORF within the 5'UTR of *ATF4* and *GCN4* can also induce translation during eIF2 α phosphorylation (17, 51). Moreover, the transcription factors C/EBP α and C/EBP β , are also regulated by a single uORF in response to eIF2 α phosphorylation (52, 53). These mechanisms are currently poorly understood and appear to be distinct from the reinitiation mechanism (17). Further studies are required to determine whether the mechanisms via these single uORFs and the *GADD34* 5'UTR share similar properties.

The finding that both transcriptional and translational mechanisms control *GADD34* expression suggests that *GADD34* is tightly regulated. Indeed, overexpression of *GADD34* leads to apoptosis in some cell lines (54–56), and forced expression of the C-terminal region of *GADD34* in mice causes dysfunction in glucose metabolism in the liver (53). Our results indicate that uORF2 regulation within the 5'UTR maintains low basal *GADD34* expression during unstressed conditions and is important in the translational induction for optimal expression during cellular stress. Given that uORFs are predicted in ~25% of all 5'UTRs, further characterization of *GADD34* uORF translational control is warranted (57). The elucidation of the *GADD34* 5'UTR mechanism may shed light on how other stress-induced mRNAs are translated during eIF2 α phosphorylation.

Acknowledgments—We thank Bruno Fonseca and Julianne Garrey for critical reading of the manuscript. Maria Hatzoglou kindly provided the mouse Hepa cells and Jim Johnson provided the CHOP antibody.

REFERENCES

1. Ron, D., and Harding, H. P. (2007) in *Translational Control in Biology and Medicine* (Mathews, M. B., Sonenberg, N., and Hershey, J., eds) pp. 345–368, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Dever, T. E., Dar, A. C., and Sicheri, F. (2007) in *Translational Control in Biology and Medicine* (Mathews, M. B., Sonenberg, N., and Hershey, J., eds) pp. 319–344, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Kostura, M., and Mathews, M. B. (1989) *Mol. Cell. Biol.* **9**, 1576–1586
4. Han, A. P., Yu, C., Lu, L., Fujiwara, Y., Browne, C., Chin, G., Fleming, M., Leboulch, P., Orkin, S. H., and Chen, J. J. (2001) *EMBO J.* **20**, 6909–6918
5. Chen, J. J., Throop, M. S., Gehrke, L., Kuo, I., Pal, J. K., Brodsky, M., and London, I. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7729–7733
6. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) *Cell* **68**, 585–596
7. Harding, H. P., Zhang, Y., and Ron, D. (1999) *Nature* **397**, 271–274
8. Tardif, K. D., Mori, K., and Siddiqui, A. (2002) *J. Virol.* **76**, 7453
9. Harding, H. P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D. D., and Ron, D. (2001) *Mol. Cell* **7**, 1153–1163
10. Schneider, R., and Sonenberg, N. (2007) in *Translational Control in Biology and Medicine* (Mathews, M. B., Sonenberg, N., and Hershey, J., eds) pp. 401–432, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

³ E. Jan, unpublished data.

11. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001) *Mol. Cell* **7**, 1165–1176
12. Alwine, J. C. (2008) *Curr. Top. Microbiol. Immunol.* **325**, 263–279
13. Pestova, T. V., Lorsch, J. R., and Hellen, C. U. (2007) in *Translational Control in Biology and Medicine* (Mathews, M. B., Sonenberg, N., and Hershey, J., eds) pp. 87–128, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Proud, C. G. (2005) *Semin. Cell Dev. Biol.* **16**, 3–12
15. Rowlands, A. G., Panniers, R., and Henshaw, E. C. (1988) *J. Biol. Chem.* **263**, 5526–5533
16. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) *Mol. Cell* **6**, 1099–1108
17. Lu, P. D., Harding, H. P., and Ron, D. (2004) *J. Cell Biol.* **167**, 27–33
18. Zhou, D., Palam, L. R., Jiang, L., Narasimhan, J., Staschke, K. A., and Wek, R. C. (2008) *J. Biol. Chem.* **283**, 7064–7073
19. Watatani, Y., Ichikawa, K., Nakanishi, N., Fujimoto, M., Takeda, H., Kimura, N., Hirose, H., Takahashi, S., and Takahashi, Y. (2008) *J. Biol. Chem.* **283**, 2543–2553
20. Vattem, K. M., and Wek, R. C. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 11269–11274
21. Jackson, R. J., Kaminski, A., and Poyry, T. A. A. (2007) in *Translational Control in Biology and Medicine* (Mathews, M. B., Sonenberg, N., and Hershey, J., eds) pp. 197–224, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Kenzelmann, M., Maertens, S., Hergenahhn, M., Kueffer, S., Hotz-Wagenblatt, A., Li, L., Wang, S., Ittrich, C., Lemberger, T., Arribas, R., Jonnakuty, S., Hollstein, M. C., Schmid, W., Gretz, N., Gröne, H. J., and Schütz, G. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104**, 6164
23. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calton, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) *Mol. Cell* **11**, 619–633
24. Novoa, I., Zeng, H., Harding, H., and Ron, D. (2001) *J. Cell Biol.* **153**, 1011–1022
25. Ma, Y., and Hendershot, L. M. (2003) *J. Biol. Chem.* **278**, 34864–34873
26. Novoa, I., Zhang, Y., Zeng, H., Jungreis, R., Harding, H. P., and Ron, D. (2003) *EMBO J.* **22**, 1180
27. Marciniak, S., Yun, C., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H., and Ron, D. (2004) *Genes Dev.* **18**, 3066–3077
28. Johannes, G., Carter, M. S., Eisen, M. B., Brown, P. O., and Sarnow, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 13118–13123
29. Jan, E., Kinzy, T. G., and Sarnow, P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15410–15415
30. Jan, E., and Sarnow, P. (2002) *J. Mol. Biol.* **324**, 889–902
31. Fernandez, J., Yaman, I., Sarnow, P., Snider, M. D., and Hatzoglou, M. (2002) *J. Biol. Chem.* **277**, 19198–19205
32. Grant, C. M., Miller, P. F., and Hinnebusch, A. G. (1994) *Mol. Cell. Biol.* **14**, 2616–2628
33. Kozak, M. (1987) *Mol. Cell. Biol.* **7**, 3438–3445
34. Child, S. J., Miller, M. K., and Geballe, A. P. (1999) *J. Biol. Chem.* **274**, 24335–24341
35. Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867–19870
36. Kozak, M. (1984) *Nature* **308**, 241–246
37. Kozak, M. (1986) *Cell* **44**, 283–292
38. Koritzinsky, M., Magagnin, M. G., van den Beucken, T., Seigneuric, R., Savelkoul, K., Dostie, J., Pyronnet, S., Kaufman, R. J., Weppeler, S. A., Voncken, J. W., Lambin, P., Koumenis, C., Sonenberg, N., and Wouters, B. G. (2006) *EMBO J.* **25**, 1114–1125
39. Brush, M. H., Weiser, D. C., and Shenolikar, S. (2003) *Mol. Cell. Biol.* **23**, 1292–1303
40. Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M., and Shenolikar, S. (2001) *Mol. Cell. Biol.* **21**, 6841–6850
41. Sachs, M. S., and Geballe, A. P. (2006) *Genes Dev.* **20**, 915–921
42. Kozak, M. (2001) *Nucleic Acids Res.* **29**, 5226–5232
43. Poyry, T. A., Kaminski, A., and Jackson, R. J. (2004) *Genes Dev.* **18**, 62–75
44. Poyry, T. A., Kaminski, A., Connell, E. J., Fraser, C. S., and Jackson, R. J. (2007) *Genes Dev.* **21**, 3149–3162
45. Gaba, A., Jacobson, A., and Sachs, M. S. (2005) *Mol. Cell* **20**, 449–460
46. Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A. G., and Sachs, M. S. (2001) *EMBO J.* **20**, 6453–6463
47. Fang, P., Wang, Z., and Sachs, M. S. (2000) *J. Biol. Chem.* **275**, 26710–26719
48. Fang, P., Spevak, C. C., Wu, C., and Sachs, M. S. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 4059–4064
49. Jan, E. (2006) *Virus Res.* **119**, 16–28
50. Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A. A., Koromilas, A. E., Zhou, L., Snider, M. D., Scheuner, D., Kaufman, R. J., and Hatzoglou, M. (2003) *Cell* **113**, 519–531
51. Mueller, P. P., and Hinnebusch, A. G. (1986) *Cell* **45**, 201–207
52. Calkhoven, C. F., Muller, C., and Leutz, A. (2000) *Genes Dev.* **14**, 1920–1932
53. Oyadomari, S., Harding, H. P., Zhang, Y., Oyadomari, M., and Ron, D. (2008) *Cell Metab.* **7**, 520–532
54. Hollander, M. C., Poola-Kella, S., and Fornace, A. J., Jr. (2003) *Oncogene* **22**, 3827–3832
55. Adler, H. T., Chinery, R., Wu, D. Y., Kussick, S. J., Payne, J. M., Fornace, A. J., Jr., and Tkachuk, D. C. (1999) *Mol. Cell. Biol.* **19**, 7050–7060
56. Hollander, M. C., Zhan, Q., Bae, I., and Fornace, A. J., Jr. (1997) *J. Biol. Chem.* **272**, 13731–13737
57. Iacono, M., Mignone, F., and Pesole, G. (2005) *Gene (Amst.)* **349**, 97–105