Molecular Cell, Vol. 6, 1099–1108, November, 2000, Copyright ©2000 by Cell Press

Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells

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Summary

Protein kinases that phosphorylate the alpha subunit of eukaryotic initiation factor 2 (eIF2α) are activated in stressed cells and negatively regulate protein synthesis. Phenotypic analysis of targeted mutations in murine cells reveals a novel role for eIF2α kinases in regulating gene expression in the unfolded protein response (UPR) and in amino acid starved cells. When activated by their cognate upstream stress signals, the mammalian eIF2 kinases PERK and GCN2 repress translation of most mRNAs but selectively increase translation of Activating Transcription Factor 4 (ATF4), resulting in the induction of the downstream gene CHOP (GADD153). This is the first example of a mammalian signaling pathway homologous to the well studied yeast general control response in which eIF2α phosphorylation activates genes involved in amino acid biosynthesis. Mammalian cells thus utilize an ancient pathway to regulate gene expression in response to diverse stress signals.

Introduction

Mammalian cells have several different eIF2α kinases that inhibit translation initiation and protein synthesis in response to specific stress signals. PKR activation by double-stranded RNA shuts down the hosts protein synthesis machinery, interfering with viral infections (Clemens and Elia, 1997; Samuel et al., 1997). PERK (also known as PEK) activation by malfolded proteins in the endoplasmic reticulum (ER) reduces the load on the protein folding and degradation apparatus in the organelle (Harding et al., 1999; Harding et al., 2000; Sood et al., 2000a). HRI attenuates protein synthesis in heme-deficient erythroid precursors, matching hemoglobin synthesis with iron availability (Chen and London, 1995). In cells deprived of nutrients, activation of mammalian GCN2 conserves scarce amino acids for use in essential metabolic processes (Berlanga et al., 1999; Sood et al., 2000b). The stress of toxins like arsenite, that are believed to modify protein structure, results in high levels of eIF2α phosphorylation and attenuation of protein synthesis (athough the responsible kinase(s) have not yet been identified Brostrom and Brostrom, 1998).

Our present understanding of mammalian eIF2α kinases emphasizes their role as stress-activated inhibitors of protein synthesis. In yeast, however, the single eIF2α kinase, Gcn2p, can control not only rates of translation initiation but also up-regulates a coordinately expressed set of genes involved in amino acid biosynthesis and metabolism (the so-called "general control" response (Hinnebusch, 1994; Hinnebusch, 1996). eIF2α, phosphorylated on serine 51, is an essential intermediate in this pathway for regulated gene expression. Phosphorylated eIF2α inhibits eIF2B, the guanine nucleotide exchange factor for the eIF2 complex and decreases assembly of 43S initiation complexes (Clemens, 1996). Lowered level of 43S initiation complexes result in lower rates of translation initiation on most mRNA and reduced synthesis of most proteins. The *GCN4* mRNA is an exception; under normal conditions this mRNA is very inefficiently translated but under conditions of limiting 43S complex formation, initiation at the protein coding AUG of this mRNA paradoxically increases, resulting in increased biosynthesis and accumulation of Gcn4p. Gcn4p is a transcription factor that binds to and activates the target genes of this pathway (Hinnebusch, 1997).

The striking conservation in structure and function between mammalian and yeast eIF2α and eIF2α kinases (Hinnebusch, 1994; de Haro et al., 1996), led us to question if the above mechanism for transcriptional activation of gene expression might also be conserved. We find that in addition to regulating protein synthesis rates, mammalian eIF2α kinases also participate in regulating stress-induced gene expression at the transcriptional level. They do so by a translational mechanism related to that employed in the yeast general control response. This hitherto unrecognized mammalian pathway for regulated gene expression is implicated in the cellular response to malfolded proteins in the endoplasmic reticulum, in the response to amino acid starvation, and is likely activated by other upstream signals.

Results

The cellular response to unfolded proteins in the ER (UPR) has two known functional components: inhibition of protein synthesis and activation of gene expression. Two distinct signaling pathways mediated by IRE1α & β and ATF6 have been implicated in activating gene expression, whereas PERK was believed to be responsible for translational regulation in the UPR (reviewed in Kaufman, 1999). In the absence of PERK, cells experience more ER stress and are hypersensitive to its lethal effects (Harding et al., 2000). In addition, IRE1α is highly active in *Perk-/-* cells (Harding et al., 2000). We expected therefore that ER-stress responsive genes would be inducible in stressed *Perk-/-* cells. Surprisingly, the gene encoding the ER stress-inducible transcription factor CHOP/GADD153 (Wang et al., 1996; Wang et al., 1998b)

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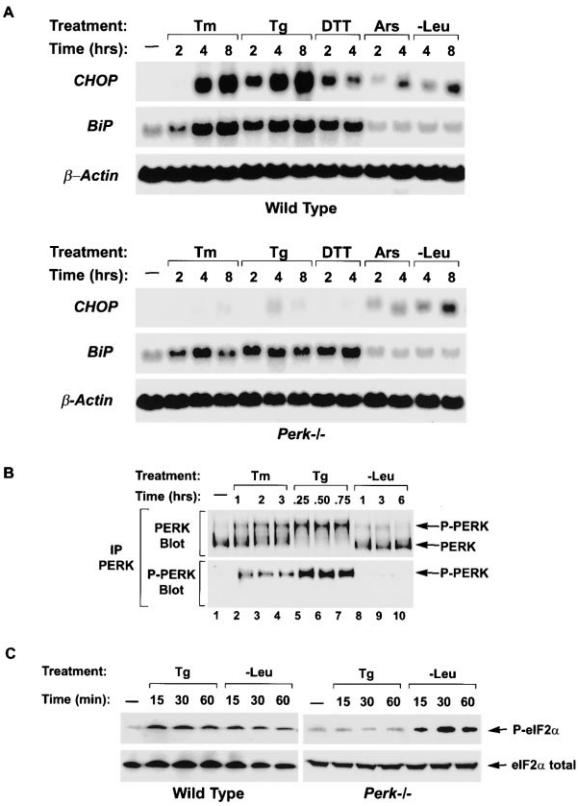

Figure 1. PERK is required for *CHOP* gene expression during ER stress

(A) Northern blot of RNA from *Perk*-/- and wild-type mouse embryonic stem cells treated with agents that promote ER stress: thapsigargin (Tg), tunicamycin (Tm) and dithiothreitol (DTT), or conditions that induce *CHOP* without causing ER stress: arsenite (Ars) and leucine deprivation (-Leu), or left untreated. The blot was hybridized sequentially with radiolabeled *CHOP*, *BiP* and β-*Actin* cDNA probes.
(B) Immunoblot analysis of anti-PERK immunoprecipitates. The blot in the top panel was reacted with antiserum that detects both non-phosphorylated (inactive) PERK and the phosphorylated (activated) form of the protein (P-PERK). The blot in the bottom panel was reacted with an antiserum directed against the phosphorylated, activate form of the protein. The cells were treated as in (A).
(C) Top panels, immunoblot with antiserum specific to eIF2α phosphorylated on serine 51 (P-eIF2α). Bottom panels, immunoblot with antiserum reactive with all forms of eIF2α (total eIF2α). Mouse embryonic stem (ES) cells with wild-type, or *Perk*-/- genotype were treated with 200 nM thapsigargin (Tg), deprived of leucine (-Leu) or left untreated for the indicated period of time.

was not induced by ER stress in *Perk*-/- cells (Figure 1A). The increase in BiP mRNA confirmed that the mutant cells were able to mount a UPR (Figure 1A), a conclusion that is further supported by the observation that IRE1α is activated by ER stress in the mutant cells (Harding et al., 2000). Nonetheless *BiP* mRNA induction was reproducibly attenuated in ER-stressed *Perk*-/- cells, indicating that *CHOP* and *BiP* share upstream regulators. The residual activation of *BiP* mRNA by ER stress in *Perk*-/- cells suggests that other signaling pathways upstream of BiP are able to compensate for lack of *Perk*, whereas *CHOP* induction is highly *Perk*-dependent.

The *CHOP* gene is also activated by other stressful stimuli, such as exposure to arsenite and amino acid deprivation (Bruhat et al., 1997; Fawcett et al., 1999, and Figure 1A). We noted that these inducers of *CHOP* also promote eIF2α phosphorylation (Duncan and Hershey, 1987; Scorsone et al., 1987; Brostrom and Brostrom, 1998). *CHOP* induction in response to arsenite and amino acid deprivation was unimpaired in the *Perk*-/- *cells*, consistent with the observation that neither stimulus activates the PERK kinase (Harding et al., 1999, and Figure 1B). The correlation between *CHOP* induction and eIF2α phosphorylation was further supported by the observation that *PERK* is required for eIF2α phosphorylation specifically by ER stress (Harding et al., 2000), but not by amino acid starvation (Figure 1C) or arsenite (Harding et al., 1999).

Recently, a mammalian eIF2α kinase similar in sequence to yeast Gcn2p has been identified (Berlanga et al., 1999; Sood et al., 2000b). We hypothesized that it might be responsible for *CHOP* activation in amino acid starved cells. First we sought to determine if the kinase was specifically activated by nutritional cues. We raised antisera to a phosphopeptide corresponding to the activation loop of the murine GCN2 and found that the kinase undergoes phosphorylation in its activation loop in cells deprived of leucine. This phosphorylation event was specific to amino acid deprivation as it was not observed in cells undergoing ER stress or following exposure to arsenite (Figure 2A and data not shown). Phosphorylation of GCN2 on the activation loop was also observed when CHO^{tsH1} cells, bearing a temperature sensitive mutation in leucyl-tRNA synthetase (Thompson et al., 1973), were switched to the non-permissive temperature (Figure 2B) and when proline auxotrophic mutant CHO-K1 cells were cultured in proline-free media (data not shown). These results suggest that like its yeast homologue, the trigger for mammalian GCN2 activation is likely to be uncharged tRNAs (Wek et al., 1989).

We produced mouse embryonic stem cells with a targeted mutation in *GCN2*. The mutation deletes an essential region of the kinase (amino acids 606–648, Figure 2C and 2D) and produces no detectable mRNA (Figure 2E) or protein (Figure 2F), indicating that it is a null allele. *Gcn2*+/- ES cells were converted to *Gcn2*-/- genotype by culture at high concentration of G418. Phosphorylated eIF2α accumulated to wild-type levels in ER stressed *Gcn2*-/- cells, but eIF2α remained largely unphosphorylated when these cells were deprived of leucine (Figure 3A). Wild-type cells phosphorylated eIF2α in response to both stressful challenges (Figure 1C). Phosphorylated eIF2α inhibits translation initiation, causing dissociation of polyribosomes and accumulation of monoribosomes and ribosomal subunits in stressed cells (Clemens, 1996). Analysis of ribosomal profiles revealed that translation initiation was attenuated in thapsigargin-treated *Gcn2*-/- cells, but remained abnormally elevated when these cells were deprived of leucine (Figure 3B). These results indicate an essential role for GCN2 in eIF2α phosphorylation and translation regulation specifically in amino acid starved mammalian cells.

Next we examined the impact of defective eIF2α phosphorylation on downstream signaling in amino acid starved cells. *Gcn2*-/- cells induced *CHOP* mRNA normally in response to ER stress but no induction was observed in response to leucine-starvation (Figure 3C). These results indicate that GCN2 specifically couples

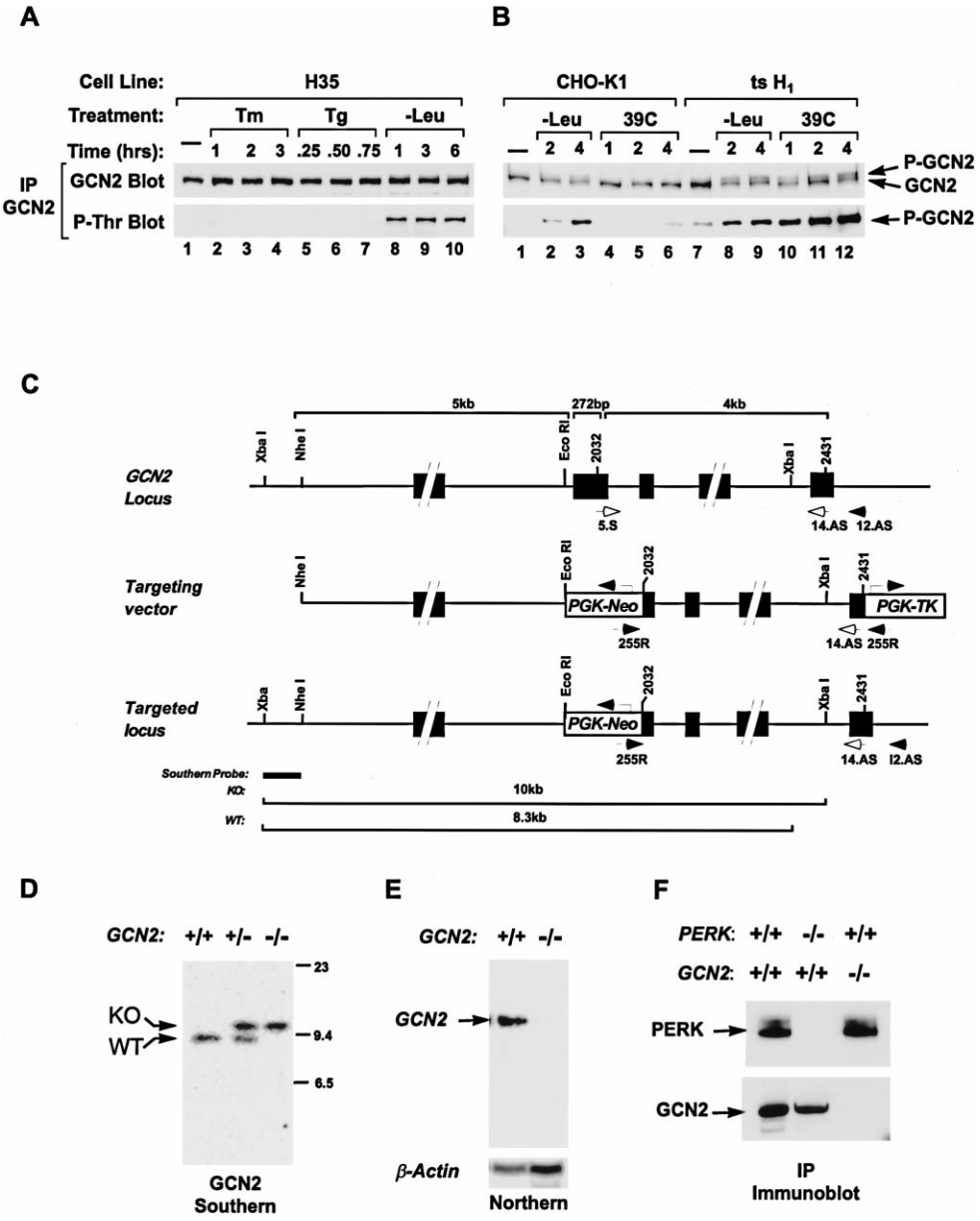

Figure 2. Characterization of an induced mutation in the mouse *Gcn2* locus

(A) Immunoblot of GCN2 immunoprecipitated from H35 rat hepatoma cells treated with tunicamycin (Tm), thapsigargin (Tg), deprived of leucine (-Leu) or left untreated for the indicated period of time. Immunoblots of the immunoprecipitated GCN2 were probed with antisera reactive with all forms of GCN2 (top panel) or with antisera to a phospho-threonine containing peptide from the activation loop of GCN2 that recognizes only the activated form of the protein (bottom panel). The phosphorylated (activated) form of the GCN2 (P-GCN2) migrates slower than the inactive form.

(B) Immunoblot of GCN2 immunoprecipitated from wild-type cells (CHO-K1) or a derivative mutant line (tsH1) that contains a temperature sensitive mutation in the leucyl-tRNA synthetase. Cells were left untreated or deprived of leucine at the permissive temperature (32°C) or shifted to the non-permissive temperature of 39°C. Immunoblots were reacted with anti-GCN2 sera or the anti-phospho-GCN2 sera as in (A).

(C) The region of the *Gcn2* locus targeted in the mutant allele. Exons are depicted by black boxes and introns by lines. The interrupted black boxes indicate that the sequence at exon-intron boundaries are not known. The arrows refer to oligonucleotides used to amplify the genomic DNA and validate the construction of the targeting vector. The boundaries of the 5' and 3' homology arms as well as the 272 base-pair replacement with the PGK-Neor cassette, are indicated. Numbering is according to residues in the mouse *Gcn2* cDNA (EMBL nucleotide accession AJ243533).

(D) Southern blot analysis of genomic DNA from wild-type, *Gcn2*+/- and *Gcn2*-/- ES cells digested with *XbaI* and hybridized to the *XbaI-NheI* fragment shown in (A). The position of the hybridizing fragments of the wild-type and mutant alleles is indicated.

(E) Northern blot analysis of 5 μg Poly A+ RNA from wild-type and *Gcn2*-/- ES cells hybridized to the full-length GCN2 and β-Actin cDNAs.

(F) Immunoblot analysis of immunoprecipitated GCN2 and PERK from ES cells with the indicated genotypes.

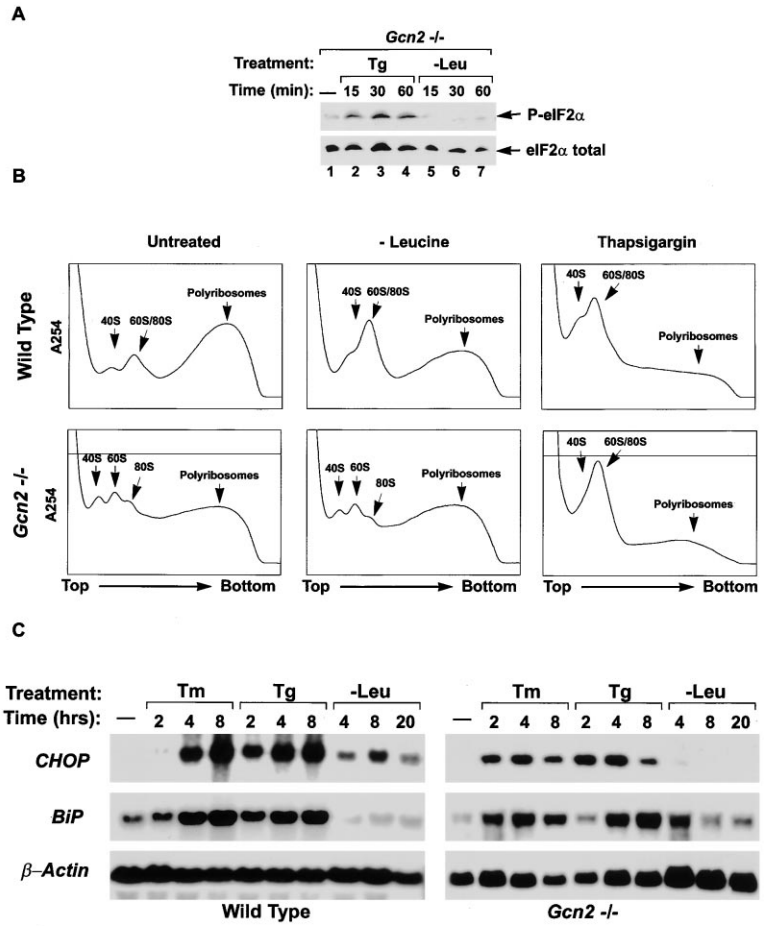

Figure 3. GCN2 is required for eIF2α phosphorylation, translation control and *CHOP* expression in amino acid starved cells

(A) Immunoblot revealing eIF2α phosphorylation in *Gcn2-/-* cells. Top panel, immunoblot with antiserum specific for eIF2α phosphorylated on serine 51 (P-eIF2α). Bottom panel, immunoblot with antiserum reactive with all forms of eIF2α in the cells (eIF2α total). Cells were treated with 200 nM thapsigargin (Tg), deprived of leucine (-Leu) or left untreated for the indicated period of time as in Figure 1C.

(B) Polyribosome profiles (absorbence at 254 nm) in cell lysates fractionated by sucrose density ultracentrifugation. ES cells of the indicated genotype were treated with thapsigargin, deprived of leucine, both for 30 min, or remained untreated. The position of the polyribosomes and ribosomal subunits is indicated. Note that the increase in ribosomal subunits apparent in leucine starved wild-type cells is absent in the *Gcn2-/-* cells.

(C) Northern blot analysis of wild-type and *Gcn2-/-* ES cells treated for the indicated period of time with tunicamycin (Tm), thapsigargin (Tg) or deprived of leucine (-Leu). The blot was sequentially hybridized to the *CHOP*, *BiP* and β-*Actin* labeled cDNAs and exposed for autoradiography.

nutritional stress to eIF2α phosphorylation and activation of *CHOP* gene expression. Together with experiment presented in Figure 1, these results suggest a correlation between eIF2α phosphorylation and *CHOP* gene activation.

In yeast, eIF2α kinase-dependent activation of gene expression is a result of derepression of *GCN4* and production of the transcription factor Gcn4p (Hinnebusch, 1996). The 5′ untranslated region of the constitutively expressed *GCN4* mRNA contains several short upstream open reading frames (uORFs). These are arranged in a configuration that favors repression of translation initiation at the protein coding AUG. Amino acid starvation, activation of Gcn2p, eIF2α phosphorylation and the attendant decrease in 43S initiation complexes, results in conditions favoring re-initiation at the protein coding AUG and synthesis of Gcn4p in stressed yeast (Mueller and Hinnebusch, 1986; Abastado et al., 1991; Dever et al., 1992). To identify mammalian transcription factors that might be regulated by a similar translational mechanism and that might activate *CHOP*, we constructed a database of ~7,000 known transcription factors and searched it for uORF-containing genes (Schapira et al., 2000). One of the genes identified, Activating Transcription Factor 4 (ATF4 Hai et al., 1989), stood out as a good candidate. The uORFs in the *ATF4* 5′ UTR are conserved from mammals to invertebrates (Figure 5E). *ATF4* mRNA is ubiquitously expressed, whereas the protein is present only at very low levels (Vallejo et al., 1993) and hypoxia, which induces eIF2α phosphorylation (Paschen and Doutheil, 1999), increases ATF4 protein without a concomitant increase in mRNA (Yukawa et al., 1999). Lastly, ATF4 has recently been shown to activate the *CHOP* promoter in vivo (Fawcett et al., 1999).

Both ER stress and amino acid deprivation increased ATF4 protein levels in wild-type cells (Figure 4A). This increase preceded CHOP accumulation, consistent with ATF4's role as an upstream activator of the *CHOP* promoter. PERK was required for ATF4 accumulation during ER stress, but proved dispensable in amino acid starved cells, whereas GCN2 was essential for induction by amino acid deprivation, but not by ER stress (Figure 4A). The role of the two protein kinases in activating *ATF4* thus correlated with their ability to phosphorylate eIF2α.

Wild-type, *Perk-/-* and *Gcn2-/-* cells all had constitutively high levels of *ATF4* mRNA (Figure 4C, 5A and data not shown), suggesting that post-transcriptional mechanisms regulate ATF4 expression during stress. This conclusion is supported by the observation that actinomycin D treatment did not prevent the accumulation of ATF4 protein in ER-stressed or leucine-deprived cells (Figure 4B). By contrast, both CHOP protein and mRNA accumulation were completely abolished by actinomycin D (Figure 4C), consistent with an essential transcriptional step in *CHOP* induction.

To confirm the role of ATF4 in *CHOP* activation, we transfected *Perk-/-* cells with expression plasmids encoding ATF4 and the cell surface marker CD2 and co-

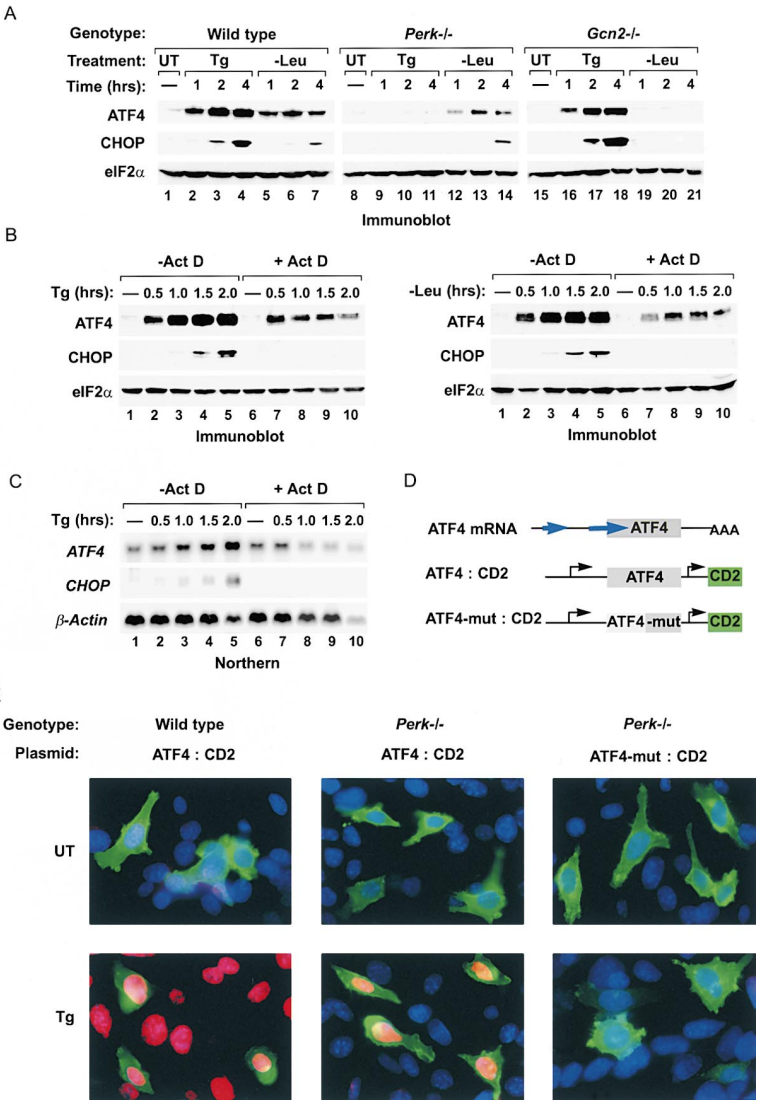

Figure 4. Post-transcriptional regulation of ATF4 expression by eIF2α kinases

(A) Immunoblot analysis of ATF4 and CHOP protein content of wild-type and *Perk-/-* and *Gcn2-/-* ES cells that had been treated with thapsigargin, placed in leucine-free medium (-Leu) or left untreated for the indicated period of time. The eIF2α immunoblot serves as a loading marker.

(B) Immunoblot of ATF4 and CHOP in wild-type cells treated with 200 nM thapsigargin (left panels), or leucine deficient media (right panel) for the indicated period of time in the presence or absence of the transcriptional inhibitor actinomycin D (Act D, 5 μg/ml added 5 min before treatment).

(C) Northern blot analysis of *ATF4* and *CHOP* mRNA performed on cells treated as in (B).

(D) and (E) Immunocytochemical detection of endogenous CHOP protein in Perk-/- mouse embryonic fibroblasts. The cells were transfected with expression plasmids encoding wild-type or truncated ATF4 protein (ATF4-mut, deleted in residues 1-252) and co-expressing a cell surface marker CD2 and stained for the presence of CD2 on the cell surface (green) and CHOP in the nucleus (red) using specific antisera. The 5' untranslated region of ATF4 was deleted from the wild-type and ATF4-mut cDNAs to insure expression of the protein. The karyophilic dye H33218 stains all the nuclei (blue). Cells were either treated with thapsigargin 200 nM for 4 hours or left untreated. Note that endogenous CHOP is absent from non-transfected Perk-/- cells but can be induced by stress in the cells transfected with the wild-type ATF4 expressing plasmid.

stained the cells for endogenous CHOP and the CD2 transfection marker. Wild-type ATF4 protein restored endogenous *CHOP* expression, whereas a mutant ATF4, lacking the N-terminal transcriptional activation domain, failed to do so (Figure 4D and 4E). These results confirm ATF4's ability to serve as an upstream activator of *CHOP*. Interestingly, we noted that *CHOP* induction was dependent on stressing the ATF4-expressing *Perk-/-* cells. This indicates a requirement for activity of other stress pathways that function independently of eIF2α phosphorylation in *CHOP* induction.

To determine the basis for the post-transcriptional stress-induced activation of *ATF4*, we examined the association of its mRNA with polyribosomes. Cell lysates were fractionated by sucrose gradient ultracentrifugation and the content of *ATF4* and β-*Actin* mRNA in individual fractions was measured by Northern blot. In unstressed cells, a substantial portion of the *ATF4* mRNA was associated with low molecular weight polyribosomes and monoribosomes (toward the top of the gradient, Figure 5A and 5B), indicating that under these conditions the mRNA is inefficiently translated. Within 20 min of the induction of ER stress, *ATF4* mRNA shifts to heavier ribosomal fractions, (toward the bottom of the gradient, Figure 5A and 5B), indicating that translation of the mRNA is de-repressed in stressed cells. *GCN4* mRNA undergoes similar re-distribution to heavier ribosomal fractions in amino acid starved yeast (Tzamarias et al., 1989). By contrast, the efficiently translated β-*Actin* mRNA was associated with the heaviest polyribosomal fractions in unstressed cells, whereas under stress it shifted to lighter fractions (Figure 5A and 5B), reflecting the inhibition of translation initiation of most mRNAs when eIF2α is phosphorylated. The divergent effect of ER stress on the association of *ATF4* and β-*Actin* mRNA with ribosomes was dependent on PERK (Figure 5A), consistent with a requirement for eIF2α phosphorylation in effecting this regulation. Not all mRNAs with uORFs are similarly regulated. For example, the *NFE2L1* mRNA, which has several uORFs, is associated with the heavy polyribosomal fraction in unstressed cells and, like β-*Actin*, shifts in position to lighter fractions in stressed cells (negative data not shown).

To determine if the above changes in mRNA-ribosome interactions correlated with ATF4 protein synthesis rates, we measured the latter by pulse-labeling with

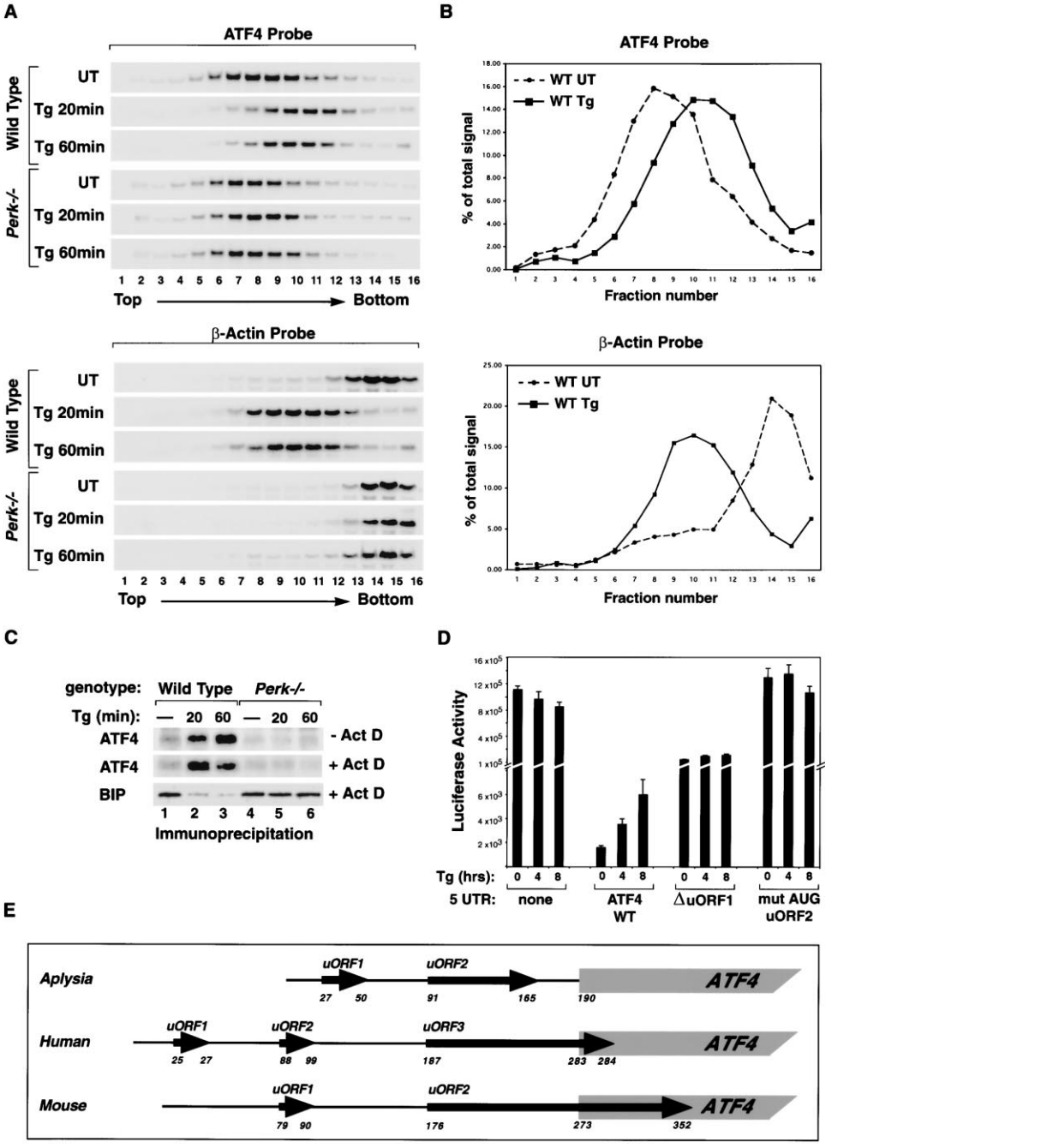

Figure 5. Stress induced, *Perk*-dependent association of *ATF4* mRNA with translating ribosomes

(A) Northern blot analysis of *ATF4* and β-*Actin* mRNA from individual fractions of sucrose gradients. Cells of wild-type or *Perk*-/- genotype were left untreated or exposed to 200 nM thapsigargin for the indicated period of time. In untreated cells, *ATF4* mRNA is found in lighter fractions than the efficiently translated β-*Actin* mRNA. ER stress causes these mRNAs to move in opposite directions on the gradient. This is dependent on the activity of the eIF2α kinase PERK.

(B) Plot of the intensity of the hybridization signal in each fraction from the experiments shown in (A). Signal intensity is expressed as its contribution (in percentage points) to the total signal integrated across the entire gradient. Shown are the fractions from the untreated (dashed line, circles) and 20 min thapsigargin treated wild-type samples (solid line, squares).

(C) In vivo metabolic pulse-labeling followed by immunoprecipitation of ATF4 and BiP from untreated and thapsigargin treated cells of the indicated genotype. Where indicated, the transcriptional inhibitor actinomycin D (5 μg/ml) was added 5 min before thapsigargin treatment.

(D) Luciferase activity of untreated and 200 nM thapsigargin-treated CHO-K1 cells transfected with the indicated reporter plasmids driven by the thymidine-kinase minimal promoter. The reporter genes consist of translational fusions of the ATF4 5′UTR and the luciferase coding region. ΔuORF1 lacks the entire ORF 1 sequence, whereas the AUG of uORF2 was replaced with an AUA sequence in mut.AUG.uORF2. Shown are the mean and SEM of experiments performed in duplicate and reproduced three times on pools of >500 clones of CHO-K1 cells, stably expressing each of the aforementioned plasmids.

(E) Diagram comparing the uORFs in the ATF4 5′UTR of mouse (NM_009716), human (HSU03712), and aplysia (ACU40851).

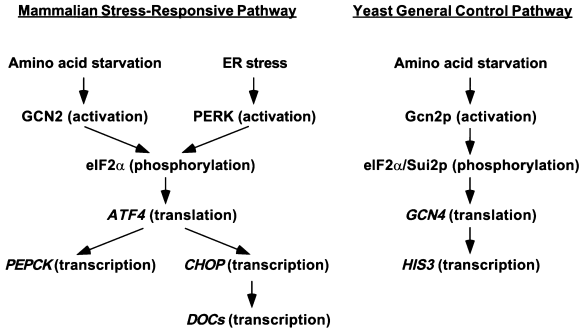

Figure 6. Comparison of genes in the yeast general control response and the homologous mammalian stress response pathway described here

The mode by which each gene is regulated is indicted in parenthesis by the gene's name. *SUI2* encodes the yeast eIF2α. *DOC*s refer to genes downstream of CHOP (Wang et al., 1998a) and *PEPCK* refers to the gene encoding the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, activated by ATF4 (Vallejo et al., 1993). *HIS3* is representative of the genes activated by yeast Gcn4p and encodes an enzyme involved in histidine biosynthesis.

^{35}S-methionine (Figure 5C). Incorporation of label into newly synthesized ATF4 was low in unstressed cells but increased (>4-fold) in response to ER stress. This increase was dependent on PERK activity and was not blocked by actinomycin D. BiP synthesis, on the other hand, was strongly inhibited at this early point of the stress response, highlighting the uniqueness of manner by which *ATF4* mRNA is translationally-regulated.

To examine more directly the role of the 5′ untranslated region in regulating *ATF4* expression, we measured the activity of reporter genes consisting of the thymidine kinase promoter driving different translational fusions between *ATF4* 5′ UTR and the luciferase reporter in stressed and unstressed cells. The translational fusions were constructed such that the initiation codon of the ATF4 coding region replaced that of luciferase. Presence of the wild-type *ATF4* 5′ UTR lowered basal expression of the reporter 500-fold and imposed on it positive regulation by ER stress. Mutating the AUG of uORF2 to AUA restored basal expression of luciferase and abolished positive regulation by ER stress. Similar, though less dramatic effects, were observed by deleting uORF1 (Figure 5D). We conclude that the evolutionarily-conserved uORFs of *ATF4* (Figure 5E), like those of yeast *GCN4*, play an important role in basal repression and post-transcriptional activation of the gene.

Discussion

These experiments reveal a pathway for regulating gene expression in mammalian cells that is homologous to the well-characterized yeast general control response to amino acid deprivation. Its components include, upstream eIF2α kinases activated by stress signals (PERK or GCN2); a transcription factor whose translation is regulated in *cis* by uORFs (ATF4); and a downstream target gene (*CHOP*) that is transcriptionally-activated by ATF4. The components of the yeast and mammalian pathways are compared in Figure 6, highlighting the evolutionary conservation in the organization of the pathway.

Despite these striking similarities in the control of *ATF4* and *GCN4* translation, we note a significant difference in the role of the uORFs in both genes: Whereas deletion of uORF1 leads to a strong decrease in Gcn2p protein synthesis (Mueller and Hinnebusch, 1986), deletion of the first uORF in the ATF4 5′UTR increases initiation at the protein coding AUG. This important difference suggests that mechanisms other than translation reinitiation may play a role in the positive regulation of *ATF4* translation by eIF2α phosphorylation. Possibilities include, leaky scanning, ribosome shunting, or internal initiation at the protein coding AUG, and these remain to be addressed experimentally.

The data presented do not exclude the formal possibility that in mammalian cells, PERK and GCN2 regulate *ATF4* translation by a mechanism that does not require eIF2α phosphorylation. Mutations in the phosphorylation site of eIF2α inhibit *GCN4* mRNA translation in amino acid starved yeast (Williams et al., 1989; Dever et al., 1992). Given the conservation in other features of the yeast and mammalian pathways, and in particular the role of the uORFs in regulated expression of ATF4, we expect this dependence on eIF2α phosphorylation to be conserved as well. A role for eIF2α phosphorylation in this pathway is directly supported by the observation that mouse fibroblasts bearing a homozygous Ser^{51}→Ala mutation in their *eIF2α* gene are also impaired in *CHOP* and BiP response to ER stress (Randy Kaufman, personal communication).

Yeast *GCN4* and mammalian *ATF4* both activate promoters of genes involved in amino acid metabolism (Hope and Struhl, 1985; Vallejo et al., 1993), suggesting that conservation in this pathway is maintained to the level of some of its most downstream components. In yeast, mutations in *GCN4* phenocopy mutations in more upstream components, suggesting a linear pathway with few bifurcations (Hinnebusch, 1997). *Atf4-/-* mice have been characterized only with respect to their most conspicuous phenotype (an impairment in eye development Tanaka et al., 1998; Hettmann et al., 2000). In the future, comparison of the phenotype of *Gcn2-/-* with the *Atf4-/-* mice may provide clues on the existence of other effectors, perhaps other transcription factors, working alongside *ATF4* in the mammalian pathway. The ATF4 target gene *CHOP* is itself a transcription factor that controls expression of a set of stress-induced target genes. These may be involved in programmed cell death or organ regeneration (Wang et al., 1998a; Zinszner et al., 1998; Sok et al., 1999). Genes like *CHOP* therefore provide a means of amplifying the signal initiated by activation of the stress-induced eIF2α kinases.

Yeast have only one known upstream activator of gene expression in the UPR, IRE1, whereas metazoans have an additional known pathway mediated by ATF6 (reviewed in Kaufman, 1999; Mori, 2000). The observation that PERK is required for *CHOP* induction during ER stress adds a third distinct pathway to the two already known to regulate gene expression in the metazoan UPR. These pathways must have different roles in the expression of target genes of the UPR. This is reflected in the observation that *CHOP* expression is completely dependent on the activity of eIF2α kinases, whereas

induction of *BiP* by ER stress is merely attenuated in the *Perk-/-* cells. In the future, it will be interesting to determine if other genes in addition to *CHOP* and *BiP* are regulated by this PERK-dependent signaling pathway.

Analysis of the rescued *Perk-/-* cells indicates that ATF4 accumulation is not sufficient for *CHOP* induction, a second stress-induced signal must be provided (Figure 4D). The nature of this signal is not known, but in the case of ER stress it may consist of activation of IRE1 or ATF6 signaling (Wang et al., 1998b; Haze et al., 1999). The requirement for a second signal may also explain differences in the degree of *CHOP's* responsiveness to ER stress, arsenite and amino acid starvation (Figure 1A). *CHOP* induction thus highlights the potential for cross talk between this pathway and other stress-induced signaling pathways.

ATF4 has been shown to play a role in memory formation (Bartsch et al., 1995), and long term potentiation of memory is also associated with the induction of *CHOP* mRNA (Matsuo et al., 2000). These observations suggest that the mammalian pathway may also respond to upstream signals unrelated to stress. It is noteworthy in this regard, that very low levels of eIF2α phosphorylation are sufficient for Gcn4p induction in yeast (Hinnebusch, 1997), suggesting that the pathway may be modulated by low level or intermittent activation of the eIF2α kinases. Yeast have only one known eIF2α kinase, Gcn2p, responsive to nutritional cues. Mammalian cells have at least 4 eIF2α kinases (PERK, GCN2, PKR and HRI) and each is activated by different signals. It is likely therefore that the pathway described here is utilized by mammalian cells to regulate gene expression in response to diverse signals.

Experimental Procedures

Gene Targeting and Cell Culture

The murine *Gcn2* gene was targeted in W4 ES cells with a positive-negative selection vector replacing the genomic region encoding amino acid residues 606–648 (Berlanga et al., 1999) that contains the critical lysine 618 required for kinase activity with a *PGK::Neo*^{r} cassette in the reverse orientation. The 5′ homology arms consisted of a 5 kb *Nhe*I-*Eco*RI genomic fragment lying 5′ of the exon encoding amino acid residues 606–748 (Berlanga et al., 1999). The 3′ homology arm consisted of the 4 kb genomic region lying 3′ of cDNA residue 2032 (amino acid 648). Clones from two independently isolated heterozygous mutant lines were selected for conversion to homozygosity in 2 mg/ml G418. Four *Gcn2-/-* lines were obtained and yielded identical results. The *Perk-/-* ES cell clones have been previously described (Harding et al., 2000). ES cells were maintained on feeders and passed two times without feeders on gelatinized plates before each experiment. H35 rat hepatoma cells were maintained in DMEM and CHO-K1 and tsH1 mutant derivative (a gift of JW Pollard) were maintained in Ham's F12 each with 10% fetal calf serum. Cells were treated with 2.5 μg/ml tunicamycin, 200 nm thapsigargin, 2 mM DTT, 100 μM sodium arsenite or placed in leucine-free media. The tsH1 and control CHO-K1 cells were maintained at the permissive temperature (34°C) or placed at the non-permissive temperature (39°C) for the indicated period of time.

Immunoprecipitation, Immunoblotting, and ATF4 Transfection

Cell lysate preparation, immunoprecipitation, and immunoblotting followed previously described procedures (Harding et al., 1999). The antisera for detecting total content of eIF2α and eIF2α phosphorylated on serine 51 have been previously described (Harding et al., 2000). The antisera reactive with total PERK (Harding et al., 1999) and the activated, phosphoryated form of the protein (Bertolotti et al., 2000) have been previously described. The antiserum to total mouse GCN2 was raised in rabbit against a GST fusion protein (residues 588–1024; Berlanga et al., 1999) and was used at a dilution of 1:5000 for immunoblotting. The serum reactive with the phosphorylated form of GCN2 was raised in rabbit against a KLH-coupled synthetic phosphopeptide: KSDPSGHLT(P)GMVGTA (corresponding to residues 890–904). When used at a dilution of 1:10,000, it recognizes only the activated protein on an immunoblot. Immunodetection of CHOP (Wang et al., 1996) and ATF4 (Vallejo et al., 1993) and the method for metabolically-labeling and immunoprecipitating ATF4 have been previously described (Vallejo et al., 1993). Cells were pretreated with thapsigargin (200 nM) for 20 or 60 min followed by a brief 10 min labeling pulse (100 μCi/ml Translabel, ICN).

Rescue of endogenous *CHOP* expression was performed by lipofectamine 2000 transfection of fibroblasts derived from day 13.5 *Perk-/-* mouse embryos with an expression plasmid encoding either full length or N-terminally truncated mouse ATF4 (ATF4-mut deleted in residues 1–252) and the CD2 cell surface marker. Thirty six hours after transfection, cells were treated with thapsigargin (200 nM, 4 hr), fixed and stained with a FITC-conjugated mouse monoclonal antibody to human CD2 and rabbit polyclonal serum to CHOP followed by texas-red conjugated goat anti-rabbit serum. The 5′ untranslated region of ATF4 was deleted from the wild-type and mutant cDNA to insure translational de-repression and expression of the proteins, which was confirmed by immunoblotting and immunocytochemistry (data not shown). The CD2 marker was used to identify transfected cells, because dual staining for ATF4 and CHOP was not feasible with the immunochemical reagents at our disposal.

Polysome Analysis and Northern Blotting

Analysis of polysomes by sucrose density gradient centrifugation of lysates from untreated, thapsigargin treated and leucine starved wild-type and *Gcn2-/-* and *Perk-/-* mutant ES cells was performed as previously described (Harding et al., 1999). Where indicated, RNA was recovered from individual fractions by phenol-chloroform extraction and ethanol precipitation and equal portions of the RNA content of each fraction were analyzed. Northern blots were hybridized to *CHOP*, *BiP*, *ATF4* and β-*Actin* cDNAs as previously described (Vallejo et al., 1993; Wang et al., 1996).

Plasmid Construction, Transfection, and Luciferase Assay

The murine 272 bp ATF4 5′UTR was isolated by RT-PCR using NIH3T3 mRNA as a template. It was cloned between the TK promoter and the luciferase gene in a TK-luciferase vector derived from pGL3-basic to create TK::ATF4UTR::Luc. The initiating AUG of ATF4 was fused in frame with luciferase. The parental TK::Luc plasmid lacking the ATF4 5′ UTR was compared with TK::ATF4UTR::Luc and two mutant derivatives. In TK::ATF4mutAUGuORF2::Luc the AUG of uORF2 was replaced with an AUA sequence. The TK::ATF4Δ-uORF1::Luc mutation deletes the *Nco*I fragment containing uORF1. Pools of >500 clones of CHO-K1 cells, stably expressing each of the aforementioned plasmids were studied. Duplicate 35 mm dishes of 80% confluent cells from each pool were treated with 200 nm thapsigargin. Cell lysis and luciferase assays were performed according to the manufacturer's instructions using the Promega luciferase assay kit.

Acknowledgments

We thank Randy Kaufman for communicating unpublished data. Supported by NIH grants ES08681 and DK47119. IN was supported by a Basque Government Fellowship. DR is a Scholar of the Leukemia and Lymphoma Society of America.

Received June 20, 2000; revised September 14, 2000.

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