Transcriptional Control of the Arginine/Lysine Transporter, Cat-1, by Physiological Stress*

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Cells respond to physiological stress by phosphorylating the α subunit of the translation initiation factor eIF2. This adaptive response inhibits protein synthesis and up-regulates genes essential for cell survival. Cat-1, the transporter for the essential amino acids, arginine and lysine, is one of the up-regulated genes. We previously showed that stress increases cat-1 expression by coordinated stabilization of the mRNA and increased mRNA translation. This induction is triggered by amino acid depletion and the unfolded protein response (UPR), which is caused by unfolded proteins in the endoplasmic reticulum. We show here that cat-1 gene transcription is also increased by cellular stress. Our studies demonstrate that the cat-1 gene promoter/regulatory region is TATA-less and is located in a region that includes 94 bases of the first exon. Transcription from this promoter is stimulated 8-fold by cellular stress. An amino acid response element within the first exon is shown to be required for the response to amino acid depletion but not to the UPR. The stimulation of transcription by amino acid depletion requires activation of GCN2 kinase, which phosphorylates eIF2α. This phosphorylation also induces translation of the cat-1 mRNA, demonstrating that stress-induced transcriptional and translational control of cat-1 are downstream targets of a signaling pathway initiating with eIF2α phosphorylation. Our studies show that the increase in cat-1 gene expression by cellular stress involves at least three types of coordinate regulation: regulation of transcription, regulation of mRNA stability, and regulation of mRNA translation.

Mammalian cells have response mechanisms that allow cells to survive amino acid limitation (1, 2). These mechanisms are largely mediated by the GCN2 kinase, which phosphorylates the translation initiation factor, eIF2α (3, 4). This phosphorylation spares amino acids by inhibiting global protein synthesis while it also increases the expression of genes that are essential for surviving amino acid limitation. One of these genes is cat-1, which encodes the high-affinity transporter for the essential cationic amino acids, Arg and Lys (5). We have previously shown that expression of the cat-1 gene is increased by amino acid starvation (6). Cat-1 gene expression is also induced by the unfolded protein response (UPR), which is triggered by the accumulation of unfolded proteins in the endoplasmic reticulum (ER) (7). This stress stimulates phosphorylation of eIF2α through PERK kinase and induces other signaling pathways as well (8).

Our previous studies have documented regulation of cat-1 gene expression at two levels. First, the turnover of cat-1 mRNA is slowed by stress, leading to increased mRNA levels (9, 10). Second, cat-1 mRNA is subject to translational control (11). The 5′ leader of the cat-1 mRNA contains an internal ribosome entry site (IRES) (11, 12). Translation from this IRES is stimulated by amino acid starvation and the UPR (12, 13). This translational control is mediated by phosphorylation of eIF2α and translation of a small upstream ORF (uORF) within the mRNA leader (12).

Because the expression of important cell defense genes is regulated at many levels (8), we also wished to know whether transcription of the cat-1 gene is regulated by cellular stresses such as amino acid starvation. In yeast, amino acid starvation stimulates the transcription of many genes by a well-characterized mechanism involving the regulator GCN4 (14). The situation is less well studied in mammalian cells, with only two genes, asparagine synthetase (AS) and C/EBP homologous protein (CHOP), studied in detail at the molecular level (15–17). The transcription of these genes is stimulated by both amino acid starvation and the UPR. The promoter regions of these genes contain an amino acid response element (AARE) in their 5′-untranslated regions that is required for the enhanced transcription during cellular stress (16). The stress-induced transcription of both of these genes has been associated with phosphorylation of eIF2α. It is believed that eIF2α phosphorylation stimulates the translation of transcription factor mRNAs, resulting in an increase in the transcription of stress-response genes. Enhanced translation of two transcription factor mRNAs, ATF4 and C/EBPβ, have been demonstrated (3, 18) and these proteins bind to the AS and CHOP promoters (17, 19, 20).

To gain a complete understanding of cat-1 gene expression, it is important to know whether transcription of this gene is also...
a site of regulation by cellular stress. In a previous study, we examined this issue using nuclear runoff (9) and did not find evidence for regulated transcription. However, runoff assays have failed to detect regulated transcription in other cases. For example, these assays did not detect transcriptional regulation of the AS gene by amino acid starvation, despite the fact that regulation of this promoter has been extensively studied by transfection assays (19).

Consequently, we undertook a more detailed study of the regulation of cat-1 gene transcription by cellular stress. In this study, we focused on the cloning and characterization of the cat-1 gene promoter region. The 5′-end of the cat-1 mRNA was cloned, and the first three exons were mapped. In contrast to some other stress-regulated genes (15), the cat-1 promoter/regulatory region does not contain a TATA element, and it includes 94 residues downstream of the transcription start site.

We show that the cat-1 promoter activity is regulated by stress. Amino acid starvation stimulated transcription via a mechanism that requires eIF2α phosphorylation. We also showed that an AARE in the first exon of the gene is required for this response. It is currently believed that the adaptive response of cells to physiological stress is a complex process involving several signaling pathways (4, 8, 21). Our results demonstrate that regulation of cat-1 gene expression at the level of transcription, translation, and mRNA stability is part of the adaptive response of cells to stress.

**EXPERIMENTAL PROCEDURES**

**Determination of Intron/Exon Boundaries**—Three clones were isolated from a rat genomic DNA library in a P1 vector (Genome Systems, Inc.). Two genomic fragments of 2.1 and 1.5 kb that contained the first and second exons, respectively, were isolated from these clones. These fragments were sequenced and compared with the cat-1 UTR sequence. RNase protection analysis was used to confirm the exon/intron boundaries.

**Primer Extension Analysis of RNA**—Primer extension was carried out using Improm II Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's protocols. A 26-base oligonucleotide (5′-CAGAAAGGGCTCATACCTGCTAGCT-3′) complementary to residues 157–182 upstream of the first cat-1 mRNA initiation codon was used as the primer. The primer was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP, purified, and extended using 20 μg of RNA from amino acid-fed or amino acid-depleted C6 cells. The cDNA products were purified and resolved on an 8% denaturing sequencing gel containing 8 M urea. Dideoxy sequencing reactions using the same primer were run on the gel as markers. The gels were dried and visualized using the PhosphorImager system (Amersham Biosciences, Piscataway, NJ).

**5′ Nucleos Nuclease Protection Analysis of RNA**—The DNA probe for 5′ nuclease mapping of the cat-1 gene transcription start site was a 265-bp SacI-NheI fragment from the PA1.4/UTR-wt plasmid (see Fig. 6). The fragment was isolated and 5′-end-labeled at the NheI site using T4 polynucleotide kinase and [γ-32P]ATP. The probe was coprecipitated with ethanol and RNA from C6 cells and suspended in a formamide-containing hybridization buffer. The samples were denatured by heating for 5 min at 95 °C and then hybridized overnight at 56 °C. Samples were then digested with 5′ nuclease following the instructions of the manufacturer (Promega, Madison, WI). Products were isolated by phenol-chloroform extraction and ethanol precipitation and analyzed on polyacrylamide gels along with DNA markers.

**Constructs and Transfections**—cat-1 expression vectors were generated using the pUHD10–3 vector (22) by replacing the XhoI/XbaI fragment with the chimeric cat-Luciferase (LUC) DNAs. In all constructs the LUC ATG was contained within an Ncol site as previously described (11, 12). PA1.4/UTR contained 1.4 kb of genomic DNA and the entire cat-1 UTR. Mutations in this vector were generated using PCR-directed mutagenesis. All other chimeric cat-LUC DNAs contained the cat-1 uORF in-frame with the LUC ATG. Expression of LUC activity under the control of the AS promoter was studied using pGL3 containing bases −115 to +1 of the AS promoter (19) and a construct for expressing ATRF (20) were obtained from M. Kilberg.

**Cells and Cell Culture**—Plasmid DNA was transfected into C6 rat glioma cells using the calcium phosphate technique (6). Stable mass culture cell lines were generated by cotransfecting an expression vector containing the neo gene and selecting the transfectants in 0.1% G418.

Cells were maintained in Dulbecco's modified Eagle medium/F-12 medium supplemented with 10% fetal bovine serum. Fed cells were incubated in Dulbecco's modified Eagle medium/F-12 supplemented with fetal bovine serum dialyzed against phosphate-buffered saline. Starved cells were incubated in KRB (Krebs-Ringer bicarbonate buffer) supplemented with dialyzed fetal bovine serum (6, 9). No difference in the regulation of the cat-1 gene by amino acid starvation was observed when KRB containing all amino acids was used in place of Dulbecco's modified Eagle medium/F-12 medium (6). Treatments were performed by culturing cells (5 × 105 cells/35-mm dish) for 48 h in growth medium followed by culture under fed or starved conditions for the appropriate times. Cells were incubated in media lacking a single amino acid as described previously (12) using the Select-Amine kit (Invitrogen). Cells were treated with amino acid alcohols (5 mM) in growth medium (23). Cells were subjected to ER stress by incubating in 400 nM thapsigargin (Thaps).

**Analytical Procedures**—RNAs were detected by Northern blotting using 32P-labeled DNA probes. Endogenous cat-1 mRNA was detected with a cat-1 cDNA probe that did not contain the first three exons; cat-LUC mRNAs were not detected by this probe. cat-LUC mRNAs were expressed from transfected cDNAs were detected with a LUC cDNA probe, which does not detect the endogenous cat-1 mRNA. AS was detected using a 900-bp fragment of the AS cDNA (23). 18 S ribosomal RNA was detected with a 5.9-kb fragment containing the 18 S mouse ribosomal DNA (24). Autoradiograms were scanned and quantified using National Institutes of Health Image software. LUC activity in cell extracts was assayed as previously described (25). ATF4 was detected on Western blots using a polyclonal rabbit antibody from Santa Cruz Biotechnology.

**RESULTS**

**Induction of cat-1 Gene Expression by Amino Acid Starvation**—We have previously shown that starvation of cells for all 20 amino acids causes increased levels of cat-1 mRNA (9). The most likely explanation of this finding is that starvation for essential amino acids triggers a signaling pathway that induces mRNA accumulation. However, there are other explanations, including the possibility that the induction is mediated by the decrease in osmolarity of the medium due to removal of amino acids. To prove that the increase in cat-1 mRNA during starvation is due directly to amino acid starvation, we examined the effects of removing single essential amino acids from the medium (Fig. 1A). This experiment showed that expression of the endogenous cat-1 mRNA was induced to a similar extent by removal of all amino acids or a single amino acid, suggesting that the effect is mediated by a signaling pathway triggered by starvation. Similar results were obtained for asparagine synthetase mRNA (AS), in agreement with previous studies (23). In addition, levels of glyceraldehyde-3-phosphate dehydrogenase mRNA and 18 S ribosomal RNA standards were not changed by these treatments.

As additional proof that the induction of the cat-1 mRNA is caused by a signal induced by amino acid limitation, the effects of two amino acid alcohols were examined. These compounds block protein synthesis by inhibiting the corresponding aminoacyl-tRNA synthases (26). As expected, both histinidinol and phenylalaninol induced cat-1 mRNA (Fig. 1B), demonstrating that the induction of cat-1 mRNA levels is caused by amino acid limitation, possibly through the accumulation of uncharged tRNAs.

**Characterization of the Promoter Regulatory Region of the cat-1 Gene**—Rat genomic DNA clones were isolated by PCR screening of a P1 phage genomic library using oligonucleotides from the 5′-end of the available cat-1 cDNA (27). We obtained three clones that cover this region with inserts of 70 kb each. We have previously described cloning of 225 nucleotides of the 5′-UTR using rapid amplification of cDNA ends (25). To identify the splice junctions within the cat-1 mRNA leader we used RNase protection analysis of mRNA from C6 cells and genomic
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DNA fragments (data not shown). This analysis showed that the 5′-UTR of the cat-1 mRNA contains three exons (Fig. 2A), interrupted by introns of ~15 kb each. Exon 1 contains 154 nt and exon 2 has 98 nt. Exons 1 and 2 contain the uORF, which encodes a peptide of 48 amino acids (25). The cat-1 ORF is located 80 nucleotides downstream of the stop codon of the uORF (Fig. 2). Its 5′-end is in exon 3, 18 nt from the 5′-end of the exon (Fig. 2).

The transcription start site of the cat-1 gene was determined using primer extension analysis of mRNA from C6 cells using a primer within the mRNAs. This primer gave two extension products at G residues 254 and 260 residues upstream of the cat-1 initiation codon (Fig. 2, B and D). Similar results were obtained with a second primer 3′ to the one shown in Fig. 2. Because of the very structured and GC-rich UTR, primer extension may have given prematurely terminated products. We therefore used S1 nuclease mapping to further determine the transcription initiation site. A genomic DNA fragment containing part of the first exon at its 3′-end was end-labeled and was used as a probe. A 93-nt product was protected, corresponding to a start site at an A residue 270 nt upstream of the cat-1 initiation codon (Fig. 2, C and D). Consequently, we propose that transcription of the cat-1 gene initiates at this Ala residue. The same transcription start site was identified in amino acid-depleted C6 cells (not shown).

Figure 1. Amino acid starvation and amino acid alcohols cause accumulation of endogenous cat-1 mRNA. A, cells were incubated for 4 h, and the level of 7.9-kb cat-1 mRNA determined by Northern blotting. Conditions include growth medium (CON), KRB without or with all 20 amino acids (KRB + AA). Cells were also incubated with KRB supplemented with all but the indicated amino acid. B, cells were incubated in KRB with (F) or without (S) all 20 amino acids, or with amino acids and 5 mM histidinol or phenylalaninol for 4 h. RNA levels were then analyzed by Northern blotting.

Transcription from the cat-1 Promoter Regulatory Region Is Regulated by Amino Acid Availability—The sequences upstream of the transcription start site do not contain any obvious TATA or CAAT elements, indicating that the cat-1 gene has a TATA-less promoter. However there are several GC-rich elements, including three putative Sp1 sites at positions –85, –58, and –48 (Fig. 2D) and four putative AP-2 sites at positions –189, –132, –111, and –57. The human genome project revealed that 50% of the genes have TATA-less promoters. However, very little is known about transcription initiation and regulation from these promoters. This issue is particularly important for cat-1, because we have shown that expression of this gene is stimulated by several types of cellular stress, including amino acid starvation, and the UPR (7). Consequently, experiments were performed to define the key promoter sequences and determine whether transcription of the cat-1 gene is regulated by cellular stress.

To study the cat-1 gene promoter, we prepared a chimeric vector (PA1.4/NheI) that contains 1.4 kb of genomic DNA upstream of the transcription start site and 94 bp of the first exon linked to a LUC reporter (Fig. 3A). Because this construct contains the AUG of cat-1 uORF, the cat-1 sequences were placed in frame with the LUC ORF so initiation of translation at either the cat-1 uORF or the LUC ATG will make a LUC protein. The mRNA transcribed from this vector does not contain the sequences in the 3′-UTR of the cat-1 mRNA that regulate mRNA stability (9). Therefore, changes in mRNA levels should reflect changes in the transcription rate.

A 2.4-kb RNA was expressed from this construct in stably transfected C6 cells (Fig. 3B), indicating that the PA1.4/NheI plasmid contains cat-1 promoter sequences. We have previously shown that the cat-1 mRNA leader has no promoter activity in either amino acid-sufficient or amino acid-depleted cells (see supplemental data in Ref. 11). We therefore conclude that sequences essential for promoter activity are within the 1.4-kb genomic DNA region.

To determine if transcription from the cat-1 promoter is affected by amino acid starvation, we compared mRNA levels in fed and starved cells. Amino acid starvation induced cat-1/LUC mRNA levels with kinetics similar to the endogenous cat-1 mRNA (Fig. 3B). The cat-1/LUC mRNA level showed a 6- to 8-fold increase by 3 h of starvation and then remained elevated until 12 h before declining (Fig. 3C). As a control for these studies, we examined the effect of amino acid deprivation on expression of the endogenous c-myc gene, which is also induced by amino acid deprivation (28). As expected, c-myc mRNA was also induced, although the kinetics were different from cat-1 (Fig. 3B). The level of c-myc mRNA reached a maximum after 3 h of starvation and then declined.

Does induction of cat-1/LUC mRNA levels by amino acid deprivation require ongoing protein synthesis? This question was addressed by examining the effects of the protein synthesis inhibitor, cycloheximide (Chx). Treatment with Chx alone had no effect on the cat-1/LUC mRNA level (Fig. 3D). In contrast, Chx prevented the increase in cat-1/LUC mRNA caused by amino acid limitation, demonstrating that protein synthesis is required for this accumulation. We also examined the effects of inhibiting transcription with ActD. Levels of the cat-1/LUC mRNA declined when ActD was added either alone or in combination with amino acid starvation, demonstrating that the accumulation of the mRNA requires ongoing transcription (Fig. 3D).

The increased cat-1/LUC mRNA level in amino acid-deprived cells could be due to increased transcription or to increased mRNA stability. To test the latter possibility, the rate of mRNA turnover was assessed by examining the decrease in mRNA levels in cells treated with ActD, which inhibits transcription. Cells were incubated under fed or amino acid-starved conditions for 4 h and then incubated further with ActD (Fig. 4). The level of the chimeric mRNA declined with similar kinetics in amino acid-fed and -deprived cells, showing that the turnover rate of the chimeric mRNA is not affected by starvation. Starvation also did not increase the stability of glyceraldehyde-3-phosphate dehydrogenase mRNA, which was examined as a
FIG. 2. Characterization of the promoter region and transcription start site of the cat-1 gene. **A**, boundaries of exons 1–3 in the 5'-end of the cat-1 mRNA are shown. The 3' boundary of exon 3 was not determined. The locations of the uORF, the cat-1 ORF, and the NheI site used for making promoter constructs are shown. **B**, mapping of the transcription start site of the cat-1 gene by primer extension. The location of the primer is shown in the schematic. The primer extension products (C6 RNA) were analyzed on a sequencing gel along with sequencing reactions from the same region as markers (G, A, T, and C). The two principal products (arrows) terminate at the G residues indicated by asterisks on the left and by gray arrows in panel **D**. Mapping of the transcription start site of the cat-1 gene by S1 nuclease protection assay. RNA was hybridized with the indicated probe, digested with S1 nuclease, and analyzed by gel electrophoresis. A digested sample (lane 1), size markers (lane 2), and the undigested probe (lane 3) are shown. **D**, sequence of the cat-1 promoter region and exon 1. The transcription start site (defined by S1 nuclease protection) is shown by the black arrow. The NSRE-1-like element is shown by a shaded box. This sequence also contains the uORF translation initiation codon in the mRNA leader. Putative binding sites for Sp1 and AP-2 are indicated, as are the SacI and NheI sites used for generating vectors and probes.
control. Because mRNA turnover is not affected by amino acid starvation, we conclude that the increase in cat-1/LUC mRNA during starvation is due to an increase in the rate of mRNA synthesis. This demonstrates that transcription from the cat-1 promoter is stimulated by amino acid starvation.

Transcription from the cat-1 Promoter Regulatory Region Is Regulated by the UPR—We have previously shown that cat-1 gene expression is stimulated by the UPR, a response to unfolded proteins in the ER (7). Increases in both the levels of cat-1 mRNA and in the translation of this mRNA are observed during the UPR (7). We also showed that the mechanism of induction of translation of the cat-1 mRNA during the UPR involves phosphorylation of eIF2α by PERK kinase (13).

Because the stability of the cat-1 mRNA is regulated, it was not clear whether the increase in cat-1 mRNA during the UPR is due to changes in transcription or mRNA stability. This issue was addressed by examining the effect of the UPR on cat-1/LUC mRNA levels in C6 cells stably transfected with PA1.4/NheI. Induction of the UPR by Thaps treatment, which mobilizes sequestered Ca²⁺ from the ER, caused an increase in both the endogenous cat-1 and the cat-1/LUC mRNAs (Fig. 3E). Moreover, the levels of both mRNAs increased after a lag of ~2 h. A similar increase in cat-1/LUC mRNA was seen when the UPR was induced by Glc starvation or treatment with tunicamycin (not shown), suggesting that the increased mRNA levels are due to increased transcription from the cat-1 promoter during the UPR. In agreement with data obtained by amino acid starvation, the induction of cat-1/LUC mRNA levels by the UPR was prevented by ActD and Chx (Fig. 3D), demonstrating that induction requires both protein synthesis and transcription. Overall, these data support the hypothesis that both amino acid deprivation and the UPR increase transcription from the cat-1 promoter.

cat-1 Gene Transcription and cat-1 mRNA Translation Are Controlled by Similar Signaling Pathways—We have shown that cat-1 mRNA translation is regulated by phosphorylation of eIF2α, which leads to the activation of translation from the cat-1 IRES (12). eIF2α can be phosphorylated by several stress-regulated kinases, including Gcn2, which is activated by uncharged tRNAs during amino acid starvation (29), and PERK,
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The First Exon of the cat-1 Gene Contains an Amino Acid Response Element—What sequences of the cat-1 promoter are required for regulated transcription during amino acid depletions? As a first step in addressing this question, we compared the promoter sequences of cat-1 and AS, a gene that is also regulated by amino acid deprivation and ER stress. Kilberg’s group identified a nutrient sensor response unit in the AS promoter 40–70 bp upstream of the transcription start site that is essential for mRNA induction by cellular stress (31, 32). This unit, which is composed of two nutrient sensor response elements (NSRE) separated by 11 nt, acts as a transcriptional enhancer in the AS promoter, because its function is independent of orientation and position (33). We found a sequence corresponding to one of these elements (NSRE-1; TGATGAAAC) in the cat-1 promoter 45 nt downstream of the transcription start site (Fig. 2D). This region also contains the initiator codon of the 5′-uORF. However, no sequences similar to NSRE-2 (GGTACA) were found.

We therefore tested if the NSRE-1-like sequence in the cat-1 promoter is important in the transcriptional regulation of the cat-1 gene by cellular stress. To do this we constructed a series of vectors that contain the 1.4 kb of the cat-1 genomic DNA upstream of the transcription start site as well as downstream sequences (Fig. 3). PA1.4/exon-1 contains the 154 nt of exon 1 fused to the LUC ORF. PA1.4/UTR contains the entire 5′-UTR of the cat-1 mRNA, with sequences from exons 1–3 (Fig. 6A). This vector was constructed because it contains all the elements required for transcriptional and translational control of the cat-1 mRNA (13). Amino acid starvation and Thaps treatment increased cat-1/LUC mRNA levels transcribed from PA1.4/exon-1 and PA1.4/UTR (Fig. 6). This increase was similar to that seen for PA1.4/NheI (Fig. 3). Because PA1.4/NheI has the smallest amount of cat-1 UTR sequences, these results show that the promoter/regulatory region that mediates the induction of transcription by stress is found within the PA1.4/NheI construct.

To determine the importance of the cat-1 NSRE-1-like element and the presence of the uORF in regulation of cat-1 promoter activity, point mutants in this region were generated in the PA1.4/UTR vector (Fig. 6). We have previously shown that the cat-1 mRNA leader contains an IRES whose activity is stimulated by cellular stress. This increase in IRES-mediated cat-1 translation requires translation of the uORF (11). The PA1.4/UTR vector was used, because the transcribed mRNA contains the entire 5′ leader of the cat-1 mRNA. Therefore, we were able to measure LUC expression from these vectors as an assay to monitor the transcriptional and translational control of these constructs (see Fig. 7).

To assess the role of the NSRE-1-like element in regulation of the cat-1 promoter, four point mutants were analyzed (PA1.4/UTR-mut1–4). These vectors were analyzed in either transient or stable transfectants. Transient transfections demonstrated that LUC activity expressed from these mutants in fed cells was similar to PA1.4/UTR, indicating that the NSRE-1-like element is not required for basal promoter activity (Fig. 7A). Similarly, mutant mRNA levels in amino acid-fed stable transfectants were similar to PA1.4/UTR-wt (Fig. 6B). However, the NSRE-1-like element was required for the induction of transcription during amino acid starvation. For mut1, 2, and 4, no increases in mRNA levels were seen at 3 h of starvation, and the increases were <20% of wild-type at 6 h (Fig. 6C). mut3 was less severely affected, with increases that were ~50% of PA1.4/UTR suggesting that the factors responsible for the activation of transcription are able to bind to this mutant. It should be noted that the cat-1 uORF is present in the mut1 and 2 constructs but is lost in mut3 and 4 because of mutations in the initiator ATG. Consequently, the ability of the NSRE-1-like element to regulate cat-1 transcription is independent of the role of uORF in the regulation of cat-1 mRNA translation. This conclusion was further supported by our analysis of a construct which is activated by ER stress (30). To determine if eIF2α phosphorylation regulates cat-1 gene transcription during amino acid starvation, we studied the effect of starvation on cat-1/LUC mRNA accumulation in cells expressing a dominant-negative mutant of GCN2 kinase (GCN2mut). We have previously shown that these cells cannot phosphorylate eIF2α in response to amino acid starvation, but phosphorylation induced by ER stress is not affected (13). Furthermore, we have shown that cat-1 IRES activity is not induced in these cells by amino acid starvation but is induced by ER stress. cat-1/LUC mRNA levels did not increase in the GCN2mut cells during amino acid depletion (Fig. 5). In contrast, cat-1/LUC mRNA levels showed a 4-fold increase during Thaps treatment of GCN2mut cells. These results demonstrate that eIF2α phosphorylation by GCN2 kinase is required for induction of cat-1 promoter activity during amino acid deprivation. They are also consistent with the specificity of the kinases that phosphorylate eIF2α: amino acid deprivation activates GCN2, whereas the UPR activates PERK. Interestingly, the levels of the endogenous cat-1 mRNA increased during amino acid starvation and Thaps treatment. This may be explained by our finding that this mRNA is stabilized during stress by a mechanism that involves the HuR protein (10).

A

![Diagram of Actinomycin D effect on cat-1/LUC and GAPDH mRNA](image)

**Fig. 4.** Amino acid starvation does not affect chimeric cat-1/LUC mRNA turnover. A, C6 cells stably transfected with PA1.4/NheI were incubated in either amino acid-fed or -starved media for 4 h. ActD was then added, and cells were cultured for the indicated times. Levels of cat-1/LUC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNAs were determined on Northern blots. B, quantification of the cat-1/LUC RNA levels in A from fed (○) and starved (●) cells.

which is activated by ER stress (30). To determine if eIF2α phosphorylation regulates cat-1 gene transcription during amino acid starvation, we studied the effect of starvation on cat-1/LUC mRNA accumulation in cells expressing a dominant-negative mutant of GCN2 kinase (GCN2mut). We have previously shown that these cells cannot phosphorylate eIF2α in response to amino acid starvation, but phosphorylation induced by ER stress is not affected (13). Furthermore, we have shown that cat-1 IRES activity is not induced in these cells by amino acid starvation but is induced by ER stress. cat-1/UTR mRNA levels did not increase in the GCN2mut cells during amino acid depletion (Fig. 5). In contrast, cat-1/UTR mRNA levels showed a 4-fold increase during Thaps treatment of GCN2mut cells. These results demonstrate that eIF2α phosphorylation by GCN2 kinase is required for induction of cat-1 promoter activity during amino acid deprivation. They are also consistent with the specificity of the kinases that phosphorylate eIF2α: amino acid deprivation activates GCN2, whereas the UPR activates PERK. Interestingly, the levels of the endogenous cat-1 mRNA increased during amino acid starvation and Thaps treatment. This may be explained by our finding that this mRNA is stabilized during stress by a mechanism that involves the HuR protein (10).
with a mutation in the stop codon of the uORF (PA1.4/UTR-stop), which removes the stop codon of uORF, creating an in-frame fusion between the uORF and the LUC ORF. We showed previously that this mutation abolished induction of IRES-mediated translation during amino acid starvation (12), and this finding was confirmed in Fig. 7B. It is shown here that amino acid starvation caused an increase in cat-1/LUC mRNA expression from PA1.4/UTR-stop (Fig. 6B), and the magnitude of this increase was similar to PA1.4/UTR, suggesting the independence of promoter and IRES function.

The effects of the mutations in the NSRE-1-like element on transcriptional regulation by ER stress were different than for amino acid starvation. The increase in mRNA was about half that of PA1.4/UTR type for mut2 (Fig. 6, B and D). In contrast, the increase for mut1, mut3, and mut4 was equal to or greater than that observed for PA1.4/UTR. This is in contrast to the regulation of the AS gene. Kilberg’s group has reported that the NSRE-1 in the AS promoter is required for induction by amino acid starvation and ER stress and that point mutations have similar effects on induction by the two stresses (15). However, a second element, NSRE-2, 11 bases downstream of the NSRE-1, is also essential for regulation of the AS promoter by both stresses. This element is not present within the cat-1 promoter; this difference could account for the differential regulation of the two genes.

Kilberg’s group has shown that NSRE-1 and NSRE-2 form an enhancer element that regulates transcription during stress in an orientation and position-independent manner (15). To determine if this is the case for the cat-1 NSRE-1-like element, we examined the ability of amino acid starvation to regulate transcription from a construct with the cat-1/UTR downstream of the minimal cytomegalovirus promoter (22). Amino acid starvation did not cause any significant changes in mRNA levels, suggesting that the cat-1 NSRE-1-like element is not an enhancer element (data not shown and Ref. 11). Furthermore, these data suggest that residues 44–154 within the first exon of the cat-1 gene do not contain an enhancer similar to the one found in the AS gene. We therefore conclude that the cat-1 sequence TGATGAAAC is an AARE (10, 12, 13, 29, 30).

We next evaluated the ability of the chimeric cat-1 mRNAs transcribed from the PA1.4/UTR plasmids to be translated. This was accomplished by assaying LUC activity. As previously discussed, the 5’ leader of the cat-1 mRNA contains an IRES whose activity is induced by amino acid starvation and ER stress. In contrast, cap-dependent translation is inhibited by these stresses. We therefore expected to see a stress-induced increase in LUC activity only for constructs that contain a functional IRES with an intact uORF. We first tested a control plasmid that expressed a LUC mRNA from the minimal cytomegalovirus promoter (Fig. 7A). LUC activity from this construct was reduced during starvation, reflecting the inhibition of cap-dependent translation. In contrast, amino acid starvation caused a large increase in LUC activity from PA1.4/UTR, which has the intact cat-1 promoter and encodes an mRNA with the entire cat-1 uORF. No induction was seen from the other cat-1 plasmids (Fig. 7, A and B). These are the expected results: The PA1.4/UTR-mut1–4 plasmids do not have inducible promoters (Fig. 7A), and the others (PA1.4/NheI, PA1.4/exon-1, and PA1.4/UTR-stop) have inducible promoters but have point mutations or deletions that inhibit activation of the IRES (Fig. 7B).

It should also be noted that no induction of LUC activity was seen with the vectors PA1.4/UTR-mut1 and mut2 (Fig. 7A), even though they have the uORF and a functional IRES. However, these constructs do not show an induction of mRNA levels during amino acid starvation (Fig. 6B). These data support the conclusion that induction of cat-1 expression during amino acid starvation requires induction of mRNA levels and a functional IRES. Overall, the findings in this report support the conclusion that cat-1 gene expression is regulated at several levels, including gene transcription and translation initiation.

The results in Figs. 6 and 7 suggest that the NSRE-1-like element in the cat-1 promoter has a different function than this element in the AS promoter. To explore this possibility, we characterized the ability of the transcription factor ATF4 to regulate expression from the cat1 promoter. Cellular stress has been reported to raise ATF4 levels (34), and ATF4 has been shown to bind to the AS and CHOP promoters and increase transcription of these genes (17, 20). To assess the role of ATF4 in regulation of the cat-1 promoter, we first examined the effect of amino acid starvation on ATF4 levels in C6 cells. As expected, starvation caused a transient increase in ATF4 level, with a 5-fold increase after 1 h and a decrease after 3 h (Fig. 8A). However, ATF4 overexpression did not affect expression from the cat1 promoter. Expression of LUC activity in cells transiently transfected with PA1.4/UTR was not affected by cotransfection of an ATF4 expression plasmid (Fig. 8B). In contrast, ATF4 overexpression did increase LUC activity expressed from the AS promoter, in agreement with results from Kilberg’s laboratory (20). Similar results were obtained when C/EBPβ was overexpressed in fed cells. Expression from the AS promoter was stimulated but cat-1 promoter-mediated expression was not affected (not shown).

**DISCUSSION**

In this study, we characterized the cat-1 promoter and its regulation by cellular stress. This promoter does not have a TATA box. Like many other TATA-less promoters (35, 36), cat-1 has several Sp1 and AP2 sites. Another common feature of TATA-less promoters is the presence of promoter elements downstream of the transcription start site (37, 38). In this study, we show that the AARE element downstream of the start site is required for regulation of promoter activity during stress caused by amino acid limitation.

There is limited information on the transcriptional regulation of amino acid transporter genes. The promoter for the
human cationic amino acid transporter, SLC7A7, has a TATA-less promoter with an AP-2 site upstream of the transcription start site. Moreover, like the cat-1 gene, the SLC7A7 gene has promoter/regulatory elements that are downstream of the transcription start site (39). We have shown here that the cat-1 AARE, which is found downstream of the transcription start site, modulates transcriptional activity but does not function as a promoter (supplemental data in Ref. 11).

Our studies demonstrate that transcription from the cat-1 promoter is stimulated by amino acid starvation and ER stress. This is in contrast to our previous report, which failed to demonstrate regulation using a nuclear runoff assay (9). This difference may be explained by the large size of the gene (the human gene is >80 kb). In addition, the cat-1 mRNA may be subject to nonsense-mediated decay (40), which may degrade mRNA during the in vitro runoff assay. Because of these issues, nuclear runoff may not provide accurate results with the cat-1 gene.

What elements are required for cat-1 promoter function? We show here that point mutations in the cat-1 AARE 45 residues downstream of the transcription start site abolished the stimulation of transcription by amino acid starvation. This demonstrates that the AARE is required for the stimulation of promoter activity during starvation. However, because the mutations did not have a significant effect on the transcription rate in fed cells, this element is not required for basal promoter activity. Moreover, this element does not support transcription by itself; no RNA was detected when promoter-less constructs containing the cat-1 UTR were transfected into C6 cells (supplemental data in Ref. 11). Consequently, sequences required for basal promoter activity must lie upstream of the transcription start site.

Our results demonstrate that NSRE-1-like elements can have distinct regulatory roles in different genes (CTGAT-GAAAC in cat-1 and AS, GTGATGCAAT in CHOP). This element is involved in the induction of transcription by stress in all three genes. However, in the AS gene, the element functions along with the nearby NSRE-2 element to form an enhancer. In contrast, the NSRE-2 is not found in either the CHOP or cat-1 promoters, and the cat-1 NSRE-1-like element does not function as an enhancer. Finally, Kilberg's group has shown that point mutations in the AS NSRE-1 block induction by both amino acid starvation and ER stress (31). In contrast, we found that mutations in the cat-1 NSRE-1 had different effects on induction by the two stresses. Therefore, it is likely that there are differences in the mechanisms by which these promoters are regulated.

How does amino acid starvation stimulate cat-1 gene transcription? We show that this activation requires the phosphorylation of the translation initiation factor eIF2α by GCN2 kinase, which regulates the activity of this key factor (3). eIF2α phosphorylation inhibits the translation of most messages, but some mRNAs are translated at a higher rate, including RNAs encoding the transcription factors C/EBPβ and ATF4 (3). We propose that the increased cat-1 transcription during amino acid starvation is due to the accumulation of a transcriptional
activator that interacts with the AARE. This model is consistent with our finding that the stimulation of cat-1 transcription by stress requires ongoing protein synthesis. It is also consistent with the finding that eIF2α phosphorylation increases rapidly during cellular stress and returns to control levels within 2 h (25), whereas the increase in cat-1 transcription is slower and persists after eIF2α phosphorylation has returned to the control level. The transcription factor(s) synthesized while eIF2α is phosphorylated could be responsible for the ongoing cat-1 transcription. It is unlikely that eIF2α has a direct effect on the cat-1 promoter, because eIF2α phosphorylation increases and returns to baseline levels within 2 h, whereas, cat-1 mRNA levels are still at peak levels after 12 h of amino acid starvation. It is also unlikely that the AARE interacts with a transcriptional repressor that is removed during amino acid starvation, because the basal promoter activity was not affected by mutations that inactivate the AARE. If this model were correct, these mutations should have caused an increase in the basal transcription rate.

The transcription factors involved in the stimulation of cat-1 transcription during cellular stress are not known. Two candidates are C/EBPβ and ATF4, because the translation of these proteins is stimulated by eIF2α phosphorylation and because both proteins have been implicated in the stimulation of AS gene transcription by cellular stress (19, 20). However, we failed to demonstrate binding of these proteins to an oligonucleotide containing the cat-1 AARE in vitro when this sequence was analyzed along with the AS AARE (data not shown). Furthermore, overexpression of ATF4 (Fig. 8) and C/EBPβ (not shown) in amino acid-fed cells did not induce transcription from the cat-1 promoter, in contrast to the AS and CHOP promoters. As discussed above, this difference is probably due to the sequences flanking the AARE, which are different for the three genes.

Although transcription from the cat-1 promoter was stimulated by amino acid starvation and by ER stress, the role of the AARE in regulation by these two stresses is not the same. ER stress caused accumulation of mRNA in mutants that did not respond to amino acid starvation. These data suggest that the AARE is required for the induction by amino acid starvation but not by ER stress. We therefore propose that the cat-1 promoter contains a separate ER response element. The CHOP gene also has different promoter elements that regulate transcription during amino acid starvation and ER stress (16, 17). Induction of CHOP transcription by ER stress requires an ATF composite site, which is involved in amino acid control, and a second element that is unique to ER stress (17). Interestingly, the CHOP ATF composite site contains the sequence GATGAT-GCAATG, which is similar to the AARE in the cat-1 promoter GCTGATGAAACC.

Our proposal that amino acid starvation and ER stress regulate cat-1 transcription by different mechanisms is consistent with the signaling pathways induced by these stresses. Both
stresses induce eIF2α phosphorylation via GCN2 and PERK kinases, respectively. However, the UPR also induces two other signaling pathways involving the translation factors ATF6 and XBP1 (8, 41). Activation of these factors by pathways that do not involve eIF2α phosphorylation could account for the behavior of the cat-1 AARE mutants that are activated by ER stress but not by amino acid starvation.

Finally, the studies described here support the idea that the stimulation of cat-1 protein expression during amino acid starvation requires both transcriptional and translational control of the cat-1 mRNA. We have previously shown that translation of the cat-1 mRNA is initiated by an IRES in the 5′-UTR and that translation from this IRES is stimulated by amino acid starvation. We have proposed a zipper model for the regulation of this IRES (11). The 5′-UTR of the mRNA has extensive secondary structure that inhibits activation of IRES activity. Translation of the uORF unfolds the secondary structure, allowing IRES-mediated translation initiation of cat-1 mRNA. The initiator ATG of the uORF lies within the AARE, and our studies show that increased protein expression requires both a functional AARE and an intact uORF. These findings underscore the importance of the multifactorial regulation of cat-1 gene expression. We have previously established the importance of mRNA turnover and translational control in this regulation. The present work demonstrates that transcriptional control is also a key site of regulation. All three types of regulation contribute to induced levels of cat-1 protein during stress.

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