Stress-induced Translation of ATF5 mRNA Is Regulated by the 5′-Untranslated Region*

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Yujiro Watatani, Kenji Ichikawa, Noriko Nakanishi, Maki Fujimoto, Hitoshi Takeda, Natsumi Kimura, Hidenori Hirose, Shigeru Takahashi†, and Yuji Takahashi

Department of Environmental Molecular Physiology, School of Life Science, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

Activating transcription factor (ATF) 5 is a transcription factor belonging to the ATF/cAMP-response element-binding protein gene family. We previously reported that ATF5 mRNA expression increased in response to amino acid limitation. The ATF5 gene allows transcription of mRNAs with at least two alternative 5′-untranslated regions (5′-UTRs), 5′-UTRα and 5′-UTRβ, derived from exon1α and exon1β. 5′-UTRα contains highly conserved sequences, in which the upstream open reading frames (uORFs) uORF1 and uORF2 are found in many species. This study was designed to investigate the potential role of 5′-UTRs in translational control. These 5′-UTRs differentially determined translation efficiency from mRNA. The presence of 5′-UTRα or 5′-UTRβ represses translation from the downstream ATF5 ORF. Moreover, 5′-UTRα-regulated translational repression is released by amino acid limitation or NaAsO2 exposure. This release was not seen for 5′-UTRβ. Mutation of uAUG2 in the uORF2 of 5′-UTRα restored the basal expression and abolished the positive regulation by amino acid limitation or arsenite exposure. We demonstrated that phosphorylation of eukaryotic initiation factor 2α was required for amino acid limitation-induced translational regulation of ATF5. Furthermore, arsenite exposure activated the exogenously expressed heme-regulated inhibitor kinase and induced the phosphorylation of eukaryotic initiation factor 2α in nonerythroid cells. These results suggest that translation of ATF5 is regulated by the alternative 5′-UTR region of its mRNA, and ATF5 may play a role in protecting cells from amino acid limitation or arsenite-induced oxidative stress.

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*To whom correspondence should be addressed: School of Life Science, Tokyo University of Pharmacy and Life Sciences,1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Tel.: 81-426-76-7018; Fax: 81-426-76-6811; E-mail: shigeru@ls.toyaku.ac.jp.

† The abbreviations used are: ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, CRE-binding protein; CHOP, C/EBP homologous protein; GCN2, general control nonderepressible-2; HRI, heme-regulated inhibitor; DMEM, Dulbecco’s modified Eagle’s medium; UTR, untranslated region; uORF, upstream open reading frame; MEF, mouse embryonic fibroblast; CMV, cytomegalovirus; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; NMD, nonsense-mediated decay; IRES, internal ribosome entry site; RT, reverse transcription; F, forward; R, reverse.

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Stress-induced Translational Regulation by ATF5 5′-UTR

These eIF2α kinases are general control nonderepressible-2 (GCN2), which is activated by nutritional limitation, RNA-activated protein kinase-related ER kinase/pancreatic eIF2α kinase (PERK/PEK), which is activated by protein malfolding because of ER stress, double strand RNA-activated protein kinase, which is activated in response to viral infection, and heme-regulated inhibitor (HRI), which is activated by heme deficiency in the erythroid lineage. HRI is also activated in response to oxidative stresses in Schizosaccharomyces pombe (14) and MEF cells (15). Phosphorylation of eIF2α reduces the levels of eIF2-GTP available for initiation of translation and contributes to lowered global protein synthesis coincident with induced translational expression of genes that function in the stress response.

The levels of the transcription factor ATF4 are increased in response to phosphorylation of eIF2α during amino acid limitation, ER stress, or hypoxia. Induced ATF4 expression occurs predominantly via translational control (9, 11, 16–18). Up-regulation of ATF4 induces the expression of genes that are important for the stress response to oxidative stresses in RIKEN mouse cancer cell line (Tsunaka, Japan). GCN2−/− mutant and control MEF cells were kindly provided by D. Ron (11). HeLaS3 cells were maintained in DMEM/F-12 supplemented with 10% fetal bovine serum, 2.5 mM l-glutamine, 0.12 mM l-methionine, 0.45 mM l-leucine, and 0.5 mM l-lysin-HCl or DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine. HepG2, COS7, U2OS, and MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine. The cells were kept at 37 °C in CO2 (5%), air (95%) under a humidified atmosphere. 10% dialyzed fetal bovine serum was used in amino acid limitation experiments. DNA transfection was performed using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions.

Construction of Plasmids—Hybrid genes comprising human ATF5 5′-UTR α or -β attached to the luciferase gene were constructed as follows (Fig. 2A). Human ATF5 5′-UTR α covering from +1 to +319 was amplified by RT-PCR using total RNA from HepG2 cells as a template. The primers used were ATF5-uAUG-F, 5′-CGGCCGTAGCATTCAATCCGTCTCTC-3′, and ATF5-uAUG-R, 5′-GCTGTAGACACAGGGCTGGCC-3′. Human ATF5 5′-UTR β covering from +9 to +348 was amplified by RT-PCR using total RNA from HeLaS3 as a template. The primers used were hATF5exon1dF2, 5′-GGCCGCTAGCAGGCAAGGGGGAG-3′, and ATF5-uAUG-R, 5′-GCTGTAGACACAGGGCTGGCC-3′. Each PCR product was digested with NheI. The resultant ATF5 5′-UTR α or -β fragment was inserted into pGL3-Basic vector (Promega, Madison, WI), which was digested with NcoI, blunted with T4DNA polymerase, and digested with Nhel to produce pGL3-Basic-D5′-UTR α or pGL3-Basic-D5′-UTR β. pcDNA3.1 (Invitrogen) was digested with MluI and Nhel to produce a CMV promoter/enhancer fragment. This fragment was inserted into the pGL3-Basic-D5′-UTR α or pGL3-Basic-D5′-UTR β containing from MluI and Nhel to produce a CMV promoter/enhancer fragment. This fragment was inserted into the pGL3-Basic-D5′-UTR α or pGL3-Basic-D5′-UTR β, which was digested with MluI and Nhel to produce pCMV-hATF5-5′-UTR α-LUC and pCMV-hATF5-5′-UTR β-LUC. As a 5′-UTR-less control plasmid, pGL3-CMV was constructed by inserting the above CMV promoter/enhancer fragment into pGL3-Basic digested with MluI/Nhel.

A mutation of the first ATG in the uORF1 of pCMV-hATF5-5′-UTR α-LUC at position +74 to +76 by replacement of the bases ATG with GTG to construct pCMV-hATF5-5′-UTR α mt1-LUC was introduced by PCR with the mutagenic primers ATF5-uAUG1mt-F, 5′-CCCCGGCTTTGGCTTCTGATG-3′, and ATF5-uAUG1mt-R, 5′-GCTGCTAGACACAGGGCTGGCC-3′. A mutation of the first ATG in the uORF2 of pCMV-hATF5-5′-UTR α-LUC at position +196 to +198 by replacement of the bases ATG with TTG to construct pCMV-hATF5-5′-UTR α-LUC was introduced by PCR with the mutagenic primers ATF5-uAUG2mt-F, 5′-CTGCAGCCCTTGGAGT-3′.
TTC-3', and ATF5-uAUG2mt-R, 5'-GAAGACTCCAGGGCT-GCAG-3'. To mutate both of the ATGs in uORF1 and -2, site-directed mutagenesis was performed on pCMV-hATF5-5'-UTRmt1-LUC with the primers ATF5-uAUG2mt-F and ATF5-uAUG2mt-R. All mutagenic sequences were verified by DNA sequencing. The resultant mutagenic plasmids were digested with NarI and NheI to produce ATF5-5'-UTRmt1, ATF5-5'-UTRmt2, and ATF5-5'-UTRmt3. pCMV-hATF5-5'-UTR-LUC was digested with NarI and NheI to remove the wild-type ATF5-5'-UTRα sequence. Then the ATF5-5'-UTRmt1, ATF5-5'-UTRmt2, and ATF5-5'-UTRmt3 fragments were inserted into pCMV-hATF5-5'-UTRα-LUC/NarI/NheI to produce pCMV-hATF5-5'-UTRmt1-LUC, pCMV-hATF5-5'-UTRmt2-LUC, and pCMV-hATF5-5'-UTRmt3-LUC.

The AT5uORF/luciferase in-frame fusion construct was generated as follows (Fig. 3A). An ATF5-5'-UTR in-frame fusion fragment was amplified by PCR using pCMV-hATF5-5'-UTRα-LUC as a template. The primers used were AT5-uORF-F and AT5-uORF-R-IF, 5'-P-CTGTAGCACAGTT-GCTGGGC-3'. The PCR product was digested with NcoI, which was digested with NcoI, blunted with T4DNA polymerase, and digested with NheI to produce pGL3-Basic vector (Promega, Madison, WI), which was digested with NcoI, blunted with T4DNA polymerase, and digested with NheI to produce pGL3-Basic-ATF5-5'-UTRα-IF. The CMV promoter/enhancer fragment was inserted into pGL3-Basic-ATF5-5'-UTRα-IF, which was digested with MluI and NheI to produce pCMV-hATF5-5'-UTRα-LUC-IF. The above procedure was applied to pCMV-hATF5-5'-UTRmt1-LUC-IF, pCMV-hATF5-5'-UTRmt2-LUC-IF, and pCMV-hATF5-5'-UTRmt3-LUC-IF. The cloning led to fusion between the AT5F uORF2 stop codon and the ATG codon of the luciferase coding sequence to produce uORF2 and luciferase fusion proteins.

uORF and uORF uAUG(−) expression vectors were constructed by deletion of the luciferase coding region of pCMV-hATF5-5'-UTRα-LUC and pCMV-hATF5-5'-UTRmt3-LUC. Each construct was digested with MscI/XbaI, blunted with T4DNA polymerase, and self-ligated to produce pCMV-uORF and pCMV-uORF-AUG(−). As a carrier plasmid that did not contain the uORF and luciferase sequences, the luciferase sequence was removed from pGL3-CMV by digesting the plasmid with NheI/XbaI, blunted with T4DNA polymerase, and self-ligated to produce pGL3-CMV (Luc−). Renilla luciferase plasmid, pRL-CMV (Promega, Madison, WI), and pSVβ-gal (GE Healthcare) were used as internal controls for beetle luciferase expression.

Human HRI cDNA was amplified by RT-PCR using total RNA from HepG2 cells as a template. The primers used were hHRI-F, 5'-CCGCAATTCATATCGAGGGCAACCTC-3', and hHRI-R, 5'-CACACTGGGCGGCTATCCACCGC-CCCATC-3'. The PCR product was digested with EcoRI and NotI. The resultant human HRI coding region of the cDNA was inserted into pcDNA3.1-FLAG vector (8), which was digested with EcoRI and NotI to produce pCDNA3.1-FLAG-hHRI. Expression plasmids for elf2α S51A and elf2α S51D were kindly provided by D. Ron (New York University, School of Medicine, New York).

Preparation of RNA and Quantification of Transcripts—Total RNA was isolated from cells using a GenElute Mammalian Total RNA Miniprep kit (Sigma). Five μg of total RNA was treated with 3 units of DNase I (Nippon Gene, Toyama, Japan). Reverse transcription using 0.5 μg of DNase I-treated total RNA and (dT)12-18 primers (GE Healthcare) was performed with ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The reverse-transcribed first strand cDNA was quantified with a real-time quantitative PCR system, the ABI PRISM 7700 sequence detection system, or ABI PRISM 7000 sequence detection system, using a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) or SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). The following oligonucleotides were used for the amplification of 80-, 147-, or 103-bp fragments of the cDNA corresponding to beetle luciferase, Renilla luciferase, or β-galactosidase: beetle luciferase primers, 5'-ACAAGATGATGATGCTAC-3' and 5'-CTTCACGGCG-GTCAACGAT-3'; Renilla luciferase primers, 5'-ATGGGAT-GAATGGGCTGATA-3' and 5'-GCTGCAATCTTTCCTTGTTCTT-3'; and β-galactosidase primers, 5'-GCTGCAATTAA-A-3' and 5'-GCGGACATCCTGAAACTT-CAG-3'. For the ABI PRISM 7700 sequence detection system, the samples were first heated at 50 °C for 2 min and then at 95 °C for 10 min and amplified during 40 cycles at 95 °C for 15 s and 60 °C for 1 min. For the ABI PRISM 7000 sequence detection system, the samples were first heated at 50 °C for 2 min and then at 97 °C for 10 min and amplified during 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative luciferase mRNA level was obtained as the ratio of beetle luciferase mRNA to Renilla luciferase mRNA or beetle luciferase mRNA to β-galactosidase mRNA. All values are the mean ± S.E. calculated from the results of three independent experiments.

Luciferase Assay—Cells were lysed with passive lysis buffer (Promega, Madison, WI). The cell lysates were used to determine luciferase activity with a dual-luciferase reporter assay system (Promega, Madison, WI) and Lumat LB 9501 (EG and G Berthold, Badwildbad, Germany). Luciferase activities were normalized with sea pansy luciferase activity. All values are the mean ± S.E. calculated from the results of three independent experiments.

Western Blot Analysis—Cell extracts were prepared with lysis buffer containing 100 mM Tris-HCl, pH 6.8, 70 mM urea, 2% SDS, and 1× Complete™ protease inhibitor mixture (Roche Applied Science). Protein concentration was determined using a BCA protein assay kit (Pierce). Proteins were separated by SDS-PAGE (7.5% for in-frame fusion proteins and 10% for the others) and electrophoretically transferred onto a Trans-Blot system. Membranes were blocked for 1 h at room temperature with 5% nonfat milk solution in TTBS buffer (25 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.1% Tween 20). The blots were then incubated with various antisera, including an anti-luciferase antibody (1:1000), an anti-β-galactosidase antibody (1:5000) (Promega, Madison, WI), an anti-FLAG antibody (1:5000) (Sigma), an anti-elf2α antibody (1:1000), and an anti-phospho-elf2α antibody (1:1000) (Cell Signaling, Beverly, MA) in TTBS for 40 min at room temperature, then washed three times.
Stress-induced Translational Regulation by ATF5 5’-UTR

FIGURE 1. ATF5 5’-UTRa contains a conserved uORF. A, genomic structure of the human ATF5 gene. Exon sequences are represented by open boxes and intron sequences by black lines. Coding sequences are represented by gray boxes. B, sequence alignment of the 5’-UTR region of the human, mouse, and rat ATF5 mRNA. uAUG codons are indicated. The conserved nucleotide sequences are shown by asterisks, and the nucleotide sequences of the uORFs are boxed. C, diagram comparing the uORFs in the ATF5 5’-UTR of human, mouse, rat, and Xenopus. This reveals mRNAs with a similar two-uORF configuration. White boxes represent the two uORFs. The gray box overlapping uORF2 is the ATF5-coding region. The numbers below each panel represent the nucleotide number from the 5’-end of each mRNA. D, amino acid sequence alignment of the uORF of human, mouse, and rat ATF5. The conserved amino acids are highlighted in black.

RESULTS

ATF5 5’-UTRa Contains Conserved uORFs—Fig. 1B and Fig. 1D show the 81 and 65% identity between human cDNA and mouse cDNA for ATF5 exon1α and exon1β (19). The alignment of the 5’-UTR sequences from human, mouse, and rat ATF5-R1 transcripts reveals a high degree of similarity, and the presence of two additional translation start sites, uAUG1 and uAUG2. The 5’-leader sequence for human ATF5-R1 is 319 nucleotides in length and contains two uORFs (uORF1 and uORF2) preceding the ATF5 coding region. uORF1 and uORF2 have a Kozak match of A/G at −3 and G at +4 of A(+1)UG, and these uAUGs, as well as the ATF5 initiation codon, are found within a sequence context that allows initiation of translation by ribosomes (20). The alignment of the amino acid sequences for the uORFs in these species also shows a high degree of similarity (Fig. 1D). The second uAUG delineates an ORF overlapping 53 nucleotides with the ATF5 ORF. In contrast, the relative positions of the upstream AUG and uORFs from exon1β of human and mouse ATF5 showed no significant similarities. The conservation of these 5’-UTRa uORFs among species suggests that they are functionally important for the regulation of ATF5 expression. These observations led us to focus on the effects of the uAUGs in modulating the translation of the downstream coding sequence.

ATF5 5’-UTRa Represses Translation of the Downstream Coding Sequence—We investigated whether ATF5 5’-UTRa can regulate translation of the downstream ORF. 5’-UTRa was cloned upstream of the luciferase reading frame, driven by the CMV promoter/enhancer in an expression vector (pCMV-hATF5-5’-UTRa-LUC) (Fig. 2A). The second uAUG delineates an ORF overlapping 80 nucleotides with the luciferase ORF. The construct was transfected into HeLaS3 cells. Fig. 2B shows that the luciferase activity was repressed when 5’-UTRa was present, whereas the luciferase mRNA level was not repressed. To test the generality of this phenomenon, we analyzed the effect of the 5’-UTR on the luciferase activity of COS7 cells. Translational suppression was also seen in COS7 cells. These results suggest that ATF5 translation is strongly repressed by the 5’-UTRa of the ATF5 tran-
script, and this translational regulation may be a general phenomenon in different cell lines.

**Mutations in Upstream AUUGs Abolish the Repressor Effect of the 5' UTR—**To determine the importance of the uAUGs of the 5' UTRα in regulating ATF5 translation, we constructed a series of plasmids (derived from pcMV-hATF5-5' UTRα-LUC) containing a point mutation for each uAUG or both. After transfection of these constructions into both HeLaS3 and COS7 cells, cellular extracts were prepared and assayed for luciferase activity and luciferase mRNA level (Fig. 2B). Luciferase activity was up-regulated by mutation of the AUG within the second uAUG (5' UTRαmt2), whereas mutation of the first uAUG (5' UTRαmt1) did not result in up-regulation of luciferase activity. Compared with the wild-type leader sequence, mutation of the second uAUG led to increases by 17.5-fold (HeLaS3) or 23.5-fold (COS7) in luciferase activity. When both the first and second uAUG were mutated (5' UTRαmt3), the repressor effect was also absent, because the luciferase activity was over the control level. These results show that the repression of luciferase activity because of the presence of the ATF5 5' UTRα occurs at the translational level and that uAUG2 is strongly involved in this repression. This repression of luciferase expression was also demonstrated by Western blotting (Fig. 2C). Luciferase protein expression was down-regulated by the ATF5 5' UTRα, whereas the mRNA levels were similar among all constructs.

**Translation Initiation at the Upstream ORF—**The results described above suggest that the ATF5 uORF sequence is translated after initiation of the uAUG2 codon. To test directly the possibility that the uORF could be translated, we generated a construct in which the ATF5 uORF was fused in-frame to the luciferase ORF (pcMV-hATF5-5' UTRα-LUC-IF) (Fig. 3A) and transiently transfected into cultured cells. Transfected cell extracts were purified and analyzed for luciferase expression by Western blotting using a specific antibody that recognizes luciferase. As a control β-galactosidase expression plasmid, pSV-β-
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Aromatic Acid Limitation or Arsenite Exposure Induces Translational Regulation through the 5’-UTR of ATF5 mRNA

Stress-induced eIF2α Phosphorylation Is Required for ATF5 Translational Regulation—Harding et al. (9) have shown that ATF4 expression is translationally induced via the 5’-UTR of ATF4 mRNA in response to eIF2α phosphorylation. Amino acid limitation and NaAsO₂ exposure were shown to induce eIF2α phosphorylation (13). eIF2α phosphorylation in amino acid-deprived and arsenite-treated cells was examined by immunoblotting using anti-phospho-eIF2α antibody. As shown in Fig. 5A, the extent of eIF2α phosphorylation in HeLaS3 and COS7 cells was increased by amino acid limitation and NaAsO₂ or CdCl₂ treatment.

FIGURE 5B shows that co-expression of eIF2α (S51D), which mimics the phosphorylated protein, effectively induced the translation of 5’-UTRα-LUC (4.8-fold), whereas 5’-UTRmt2-LUC (Fig. 2A) did not respond to eIF2α (S51D) expression. eIF2α (S51A), which cannot be phosphorylated, did not induce the translation of 5’-UTRα-LUC or 5’-UTRmt2-LUC. In these experiments, ATF5 mRNA levels were comparable in all COS7 cells tested. Furthermore, in wild-type MEF cells, luciferase activity was induced by leucine limitation (3.2-fold), whereas luciferase activity showed little response to leucine limitation in GCN2-/- MEF cells (0.8-fold), in which eIF2α was not efficiently phosphorylated (Fig. 5C). These results are consistent with an earlier report that ATF4 expression is induced in response to eIF2α phosphorylation and that 5’-UTR can confer this translation control when fused to a heterologous reporter gene (9).

To elucidate the mechanism by which the translation of ATF5 is induced, we investigated the translation start site shift from uORF to ATF5 ORF using an ATF5 5’-UTRα-LUC in-frame fusion construct (Fig. 3A). Fig. 5D shows that the translation initiation site was shifted from uAUG2 to the AUG of the luciferase ORF in response to NaAsO₂ exposure in COS7 cells (lanes 5 and 6). As shown in Fig. 5E, we confirmed that, when HRI protein was exogenously expressed in COS7 cells, NaAsO₂ exposures induced phosphorylation of HRI. From these findings, we conclude that eIF2α phosphorylation regulates the translation of ATF5 mRNA via its 5’-UTR in response to cellular stresses, and we suggest that in cells exposed to oxidative stresses, HRI is activated and induces eIF2α phosphorylation to up-regulate the translation of ATF5.

Alternative 5’-UTRs of Human ATF5 mRNA Have Different Translational Regulation Properties—The ATF5 gene has two exon1s, exon1α and exon1β, which are transcribed into ATF5-R1 mRNA and ATF5-R2 mRNA, respectively. The sequences of exon1α and exon1β did not show any similarity. The identity between human and mouse was 81% for exon1α and 63% for exon1β (Fig. 6A). Human ATF5-R2 mRNA harbors one initiation codon (GGGATgG) that is in a good context for
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The constructs pCMV-LUC, pCMV-hATF5-5′-UTRa-LUC, and pCMV-hATF5-5′-UTRβ-LUC were transfected into HeLaS3 cells. The luciferase activity from the luciferase transcript containing 5′-UTRβ reached only about 13% of the activity obtained from the transcript containing no UTR, which was comparable with that obtained with 5′-UTRa.

To assess whether ATF5 5′-UTRa and -β are translationally regulated in a cell-specific manner, pCMV-hATF5-5′-UTRa-LUC and pCMV-hATF5-5′-UTRβ-LUC were transfected into HeLaS3, HepG2, and U2OS cells, and luciferase mRNA levels and luciferase activities were determined. The luciferase activities from the luciferase expression plasmids containing no 5′-UTR, 5′-UTRa, or 5′-UTRβ were the same among these cell lines. We did not observe any cell-specific translational regulation, at least among these cell lines for 5′-UTRa and 5′-UTRβ.

Next we investigated whether 5′-UTRβ can modulate translation in response to amino acid limitation (Fig. 6D). HeLaS3 cells were transfected with the above three plasmids. The effect of amino acid limitation on luciferase activity and luciferase mRNA level was analyzed. As shown in Fig. 6D, although LUC activity from pCMV-hATF5-5′-UTRβ-LUC did not significantly respond to amino acid limitation (comparable with the control construct), luciferase activity from the 5′-UTRa-LUC was increased 3.8-fold by glutamine limitation and 11.4-fold by leucine limitation. These results show that ATF5 5′-UTRβ has a repressor property for translation as does 5′-UTRa, but this did not respond significantly to amino acid limitation. This suggests that these alternative 5′-UTRs have different roles in translational regulation induced by cellular stress.

**DISCUSSION**

UTRs have several roles in gene expression, including mRNA stability, mRNA localization, and translation efficiency (21). The turnover of an mRNA is mostly regulated by cis-acting elements located in the 3′-UTR, such as the AU-rich elements, which regulate mRNA decay in response to a variety of specific

**FIGURE 4. Inhibition of translation through 5′-UTRa is released by cellular stress.** A, inhibition of translation through 5′-UTRa is released by amino acid limitation. HeLaS3 cells were transiently transfected with ATF5 5′-UTRa-luciferase constructs as shown in Fig. 2. A Renilla luciferase reporter construct was used as an internal control. Twenty-four h after transfection, cells were incubated in DMEM/F-12 medium containing 15% dialyzed FBS as control (C), or in medium lacking glutamine (Gln–) or leucine (Leu–) for 6 h. Relative luciferase activity and relative mRNA level were determined as described in Fig. 2B. Results are given as fold induction, defined as the ratio of the relative luciferase activity divided by the mRNA level of deprived cells to that of nondeprived cells. Each value represents the mean ± S.E. of three independent experiments. B, inhibition of translation through 5′-UTRa is released by arsenite exposure. HeLaS3 or COS7 cells were transiently transfected with ATF5 5′-UTRa-luciferase constructs. Twenty-four h after transfection, cells were incubated in serum-free DMEM as a control (C) or in serum-free medium containing 50 μM NaAsO₂ (As) for 6 h. Luciferase assay and mRNA quantification were performed as in A. Each value represents the mean ± S.E. of three independent experiments. Fold inductions are shown as the ratio of luciferase activity to mRNA level.

translation, according to the Kozak rule. A putative uORF encoding 101 amino acids derived from the human ATF5-R2 transcript overlaps with the ATF5 ORF. The human β-specific 5′-UTR harbors two other uAUGs that are in a poor context for translation and are followed by a stop codon upstream of the initiation codon for ATF5.

To test the regulatory function of the alternative 5′-UTRs, we analyzed the translational properties of 5′-UTRβ in a transient transfection assay (Fig. 6, C and D). Human ATF5 5′-UTRβ was cloned upstream of the luciferase reading frame, driven by the CMV in an expression vector (pCMV-hATF5β-5′-UTR-LUC). The constructs pCMV-LUC, pCMV-hATF5-5′-UTRa-LUC, and pCMV-hATF5-5′-UTRβ-LUC were transfected into HeLaS3 cells. The luciferase activity from the luciferase transcript containing 5′-UTRβ reached only about 13% of the activity obtained from the transcript containing no UTR, which was comparable with that obtained with 5′-UTRa.

FIGURE 4. Inhibition of translation through 5′-UTRa is released by cellular stress. A, inhibition of translation through 5′-UTRa is released by amino acid limitation. HeLaS3 cells were transiently transfected with ATF5 5′-UTRa-luciferase constructs as shown in Fig. 2. A Renilla luciferase reporter construct was used as an internal control. Twenty-four h after transfection, cells were incubated in DMEM/F-12 medium containing 15% dialyzed FBS as control (C), or in medium lacking glutamine (Gln–) or leucine (Leu–) for 6 h. Relative luciferase activity and relative mRNA level were determined as described in Fig. 2B. Results are given as fold induction, defined as the ratio of the relative luciferase activity divided by the mRNA level of deprived cells to that of nondeprived cells. Each value represents the mean ± S.E. of three independent experiments. B, inhibition of translation through 5′-UTRa is released by arsenite exposure. HeLaS3 or COS7 cells were transiently transfected with ATF5 5′-UTRa-luciferase constructs. Twenty-four h after transfection, cells were incubated in serum-free DMEM as a control (C) or in serum-free medium containing 50 μM NaAsO₂ (As) for 6 h. Luciferase assay and mRNA quantification were performed as in A. Each value represents the mean ± S.E. of three independent experiments. Fold inductions are shown as the ratio of luciferase activity to mRNA level.
FIGURE 5. Stress-induced eIF2α phosphorylation is required for ATF5 translational regulation. A, stress induces eIF2α phosphorylation. Upper, amino acid limitation induces eIF2α phosphorylation. HeLaS3 or COS7 cells were treated with amino acid limitation for 6 h. Total proteins were separated by SDS-PAGE. The extent of eIF2α phosphorylation was detected with anti-eIF2αP antibody. Total eIF2α was determined with anti-eIF2 antibody. Lower, NaAsO2 or CdCl2 treatment induces eIF2α phosphorylation. HeLaS3 or COS7 cells were treated with NaAsO2 or CdCl2 at the indicated concentrations for 4 h. The extent of eIF2α phosphorylation was analyzed as described above. Cont, control.

B, activation of ATF5 5′-UTR-mediated translation is regulated by eIF2α phosphorylation. One day before transfection, COS7 cells were plated on 60-mm dishes. The cells were then transiently co-transfected with 1 μg of pCMV-5′-UTR-LUC alone or with expression plasmid for eIF2α S51A, or eIF2α S51D (1 μg). 0.2 μg of Renilla luciferase reporter construct was used as an internal control. Twenty-four h after transfection, relative luciferase activity and relative mRNA level were determined as described in Fig. 2B. Each value represents the mean ± S.E. of three independent experiments. Results are given as fold induction, defined as the ratio of the relative luciferase activity divided by the mRNA level of cells transfected with empty vector to that of the others.

C, phosphorylation of eIF2α is required for ATF5 translational regulation in amino acid-deprived cells. Left, wild-type or GCN2−/− MEF cells were treated with leucine limitation for 6 h and the extent of eIF2α phosphorylation was detected with anti-eIF2αP antibody. Total eIF2α was determined with anti-eIF2α antibody. Right, 1 day before transfection, wild-type or GCN2−/− mutant MEF cells were plated on 60-mm dishes. The cells were then transiently transfected with 1 μg of pCMV-5′-UTR-LUC, with 0.2 μg of Renilla luciferase reporter construct as an internal control. Four h after transfection, cells were treated with leucine limitation for 6 h and the extent of eIF2α phosphorylation was detected with anti-eIF2αP antibody. Total eIF2α was determined with anti-eIF2α antibody. Right, 1 day before transfection, wild-type or GCN2−/− mutant MEF cells were plated on 60-mm dishes. The cells were then transiently transfected with 1 μg of pCMV-5′-UTR-LUC, with 0.2 μg of Renilla luciferase reporter construct as an internal control. Twenty-four h after transfection, cells were incubated in serum-free DMEM containing 50 μM NaAsO2 for 8 h. Whole-cell lysates were then prepared and analyzed for luciferase protein by immunoblotting as described in Fig. 3B. Each value represents the mean ± S.E. of three independent experiments. Results are given as fold induction, defined as the ratio of the relative luciferase activity divided by the mRNA level of cells transfected with empty vector to that of the others.

D, translation initiation site is shifted from uAUG2 to the AUG of the luciferase (LUC) ORF. COS7 cells were transiently transfected with ATF5 5′-UTR-luciferase constructs shown in Fig. 3A (5′-UTRα, 5′-UTRβ, and 5′-UTRγ in-frame fusion). Fourty-eight h after transfection, cells were incubated in serum-free DMEM containing 50 μM NaAsO2 for 8 h. Whole-cell lysates were then prepared and analyzed for luciferase protein by immunoblotting as described in Fig. 3B. Each value represents the mean ± S.E. of three independent experiments. Results are given as fold induction, defined as the ratio of the relative luciferase activity divided by the mRNA level of cells transfected with empty vector to that of the others.

E, HRI is activated by NaAsO2 treatment of COS7 cells. One day before transfection, COS7 cells were plated on 60-mm dishes. The cells were then transiently transfected with 2 μg of FLAG-tagged HRI expression plasmid. Twenty-four h after transfection, cells were incubated in serum-free DMEM as a control or in serum-free DMEM containing 50 μM NaAsO2 for 4 h. Whole-cell lysates were then prepared and analyzed for HRI protein by immunoblotting. Two μg of β-galactosidase expression plasmid was co-transfected as an internal control, and its protein was also detected by immunoblotting.
signals, including cellular stress (22). UTRs also have a significant role in the localization of some mRNA by the mechanisms, active direct transport, local stabilization, or local entrapment of transcripts.

It is known that less than 10% of eukaryotic mRNAs contain uAUG codons within the 5′-UTR region (23). However, uAUGs are common in certain groups of genes, such as oncogenes and many other genes related to cell growth, differentiation, and stress response. Some of the mechanisms by which UTRs regulate translation are beginning to be understood. CHOP, C/EBPα, C/EBPβ, S-adenosylmethionine, and β2-adrenergic receptor have uORFs, and these repress the basal translation of the ORFs (24–27). The uORF peptide encoded in the UTR of CHOP and vigilin mRNA participates in repression of translation (24, 28). However, so far translational up-regulation of these genes has not been reported. In other cases, several mechanisms can account for translational regulation via the 5′-UTR of the transcript: ribosome re-initiation, internal ribosome entry, and leaky scanning.

Stress that induces phosphorylation of eIF2α inhibits translation and attenuates global protein synthesis. However, eIF2α phosphorylation promotes the translation of mRNA for stress-induced genes. Translational control of the yeast GCN4 gene is the first and best studied example of selective translation by eIF2α phosphorylation (23). The GCN4 is a transcription factor that activates gene expression for amino acid biosynthesis. Four uORFs in the 5′-UTR have an important role in the translational regulation of downstream coding sequences. One day before transfection, HeLaS3, HepG2, or U2OS cells were plated on 60-mm dishes and then 1.5 μg of luciferase reporter construct was transfected into the cells. Twenty-four h after transfection, cells were harvested and analyzed for luciferase activity and mRNA level as described in Fig. 2A. Each value represents the mean ± S.E. of three independent experiments.

| 5′UTR | α | β | Fold induction |
|-------|---|---|----------------|
| 5′UTR | 1.0 | 0.08 | 0.13 |
| 5′UTR | 1.0 | 0.4 | 0.06 |
| 5′UTR | 1.0 | 0.02 | 0.04 |

FIGURE 6. The alternative 5′-UTRs of human ATF5 mRNA have different translational regulation properties. A, sequence alignment of human and mouse ATF5 5′-UTR RNA: AUG sequences are boxed. These describe the sequences code for exon1β, a part of the ATF5 5′-UTR RNA sequences. The conserved nucleotide sequences are shown by asterisks. B, schematic representation of the human ATF5 5′-UTRα and β-luciferase constructs. Human ATF5 5′-UTRβ was cloned into the upstream of the luciferase coding region to produce pCMV-ATF5 5′-UTRβ-LUC. C, ATF5 5′-UTRβ represses translation of downstream coding sequences. One day before transfection, HeLaS3, HepG2, or U2OS cells were plated on 60-mm dishes and then 1.5 μg of the luciferase construct shown in B was transiently transfected, with 125 ng of Renilla luciferase reporter construct as an internal control. Twenty-four h after transfection, cells were harvested and analyzed for luciferase activity and mRNA level as described in Fig. 28. D, 5′-UTRβ does not modulate translation in response to amino acid limitation. HeLaS3 cells were transiently transfected with ATF5 5′-UTR-luciferase constructs as described in Fig. 5C. Twenty-four h after transfection, cells were treated with amino acid deprivation and analyzed for luciferase activity and relative mRNA levels as described in Fig. 28. Each value represents the mean ± S.E. of three independent experiments.
Stress-induced Translational Regulation by ATF5 5'-UTR

The adaptive response to nutritional stress involves increased translation of CAT-1 mRNA via internal ribosome entry sites (IRES) within the 5'-UTR, and this induction requires the uORF in the 5'-UTR and eIF2α phosphorylation (29). Translation of the uORF of CAT-1 5'-UTR unfolds an inhibitory structure in the 5'-UTR. It is proposed that eIF2α phosphorylation induces the synthesis of an IRES trans-acting factor, which stabilizes the inducible IRES. This conformational change yields an active IRES that allows ribosomes to initiate translation at the CAT-1 ORF (30).

Ribosome re-initiation involving upstream ORFs also regulates ATF4 mRNA translation (17, 31). The 5'-UTR of ATF4 mRNA has two conserved uORFs, uORF1 and uORF2. Scanning ribosomes that initiate translation from uAUG1 efficiently re-initiate translation at the downstream AUG, uAUG2. Under control conditions, low levels of eIF2α phosphorylation favor re-initiating ribosomes, directing them to initiate from uAUG2, which abrogates the translation of the ATF4 ORF. Under stressed conditions, high levels of eIF2α phosphorylation delay ribosome recognition of uAUG2 and favor re-initiation at the ATF4 initiation codon.

ATF5 belongs to the ATF4 subfamily of CREB/ATF transcription factors (32). The 5'-UTR regions of the ATF5-R1 mRNAs of human, mouse, and rat show high sequence similarity (Fig. 1) and the presence of two putative translation start sites (uAUG1 and uAUG2). Both the relative position and the sequence context of the uATGs of exon1α, which is suitable for efficient translation, are well conserved. These findings prompted us to investigate the role of ATF5 5'-UTR. We found that the presence of 5'-UTRα severely inhibits the translation of downstream ORFs. 5'-UTRα-dependent repression of translation is recovered by introducing a mutation in uAUG2 or exposure to stresses, including amino acid limitation and arsenite exposure. Amino acid limitation or arsenite exposure activates GCN2- or HRI-dependent eIF2α phosphorylation (13).

As shown in Fig. 5, stress-induced eIF2α phosphorylation is required for ATF5 translational regulation, although the precise mechanism for the shift of the translation initiation site remains to be elucidated.

HRI has been shown to regulate the translation of blood cell-specific globin protein (33). Recently, McEwen et al. (15) reported that HRI functions in cells other than blood cells. In this study, an exogenously expressed HRI was activated in COS7 cells. Furthermore, we demonstrated that NaAsO2 exposure induced eIF2α phosphorylation in both HeLaS3 and COS7 cells and caused a shift of translation initiation site from the uAUG to the correct ATF5 AUG site. These findings support the view that HRI may function in other types of cells besides blood cells in response to oxidative stress.

ATF4 uAUG1 has a positive function for mRNA translation (31). When the uAUG1 of ATF5 5'-UTRα was mutated, the basal translational level was down-regulated by more than 50% that for wild-type 5'-UTRα (Fig. 2B). It is noteworthy that the mt1 construct enhances expression of uORF2-luciferase and diminishes expression of luciferase (Fig. 3B). These suggest that the uAUG1 of the ATF5 5'-UTRα may have the same positive function as the ATF4 uAUG1. There is no homology between the ATF5 5'-UTRα and ATF4 5'-UTR sequence, but their function in translation has been conserved during evolution.

Although sequence similarity is moderately conserved between human and mouse exon1β sequences, neither the relative position nor the sequence context of the uAUGs are conserved. From this, we speculated that exon1β does not have any regulatory function and cannot suppress the translation of downstream ORFs. However, surprisingly, 5'-UTR derived from exon1β also inhibits translation, as does exon1α. This is supported by the in vitro observation that the presence of 5'-UTR derived from mouse exon1β hampered the translation of the ATF5 ORF (19).

Although the 5'-UTR from both exon1α and exon1β suppressed the translation of the ATF5 ORF, these two UTRs displayed differential properties in the stress response (Fig. 6). In contrast to 5'-UTRα, amino acid limitation does not release 5'-UTRβ-dependent translational repression. In some cases, 5'-UTR regulates translation in a cell type-specific manner (34–36). For example, the 5'-UTR of the branched-chain α-ketoacid dehydrogenase kinase transcript inhibits its translation in a cell type-specific manner (34). To test the cell type specificity of ATF5 5'-UTRs, we investigated 5'-UTRα and -β activity in various types of cells. Among these cells, we did not observe any cell type-specific translational regulation for either UTR. The physiological significance of the two types of 5'-UTR and their different properties remain to be elucidated.

We have reported that ATF5 mRNA expression was induced by amino acid limitation (8). In this study, we demonstrated that translational repression by 5'-UTRα is recovered by stresses, including nutrient limitation and exposure to arsenite. Recently, Sarraj et al. (37) showed that overexpression of ATF5 in HepG2 cells stimulates gene transcription via the nutrient-sensing unit of the asparagine synthetase gene. These suggest that ATF5 may be a regulator of gene expression in the response to stresses, including amino acid limitation and oxidative stress.

Eukaryotic cells have evolved several proofreading mechanisms to degrade aberrantly processed or mutant mRNAs. Eukaryotic mRNAs containing premature termination codons are subject to accelerated turnover, known as nonsense-mediated decay (NMD) (38). Inhibitors of protein synthesis have been widely used to demonstrate the role of NMD in the degradation of mRNA transcripts (39). Cycloheximide treatment increases ATF5 mRNA levels (8), indicating that NMD may be involved in the stability of ATF5 mRNA. In this study, we have identified two putative uORFs in the 5'-UTRα of ATF5 mRNA, and studies are ongoing in our laboratory to determine whether NMD recognizes the premature stop codon derived from upstream AUG and regulates stress-responsive gene expression via ATF5 5'-UTRα. The identification of this pathway provides a new area of research into stress responses in mammalian cells.

The molecular mechanism by which dietary protein intake and oxidative stress affect gene regulation is important for the regulation of physiological functions of mammalian cells. Mammalian cells adapt to amino acid limitation or oxidative stress by regulating numerous genes. Exploring the mechanisms that regulate gene expression under stresses, including amino acid limitation and oxidative stress, will increase our...
understanding of metabolic regulation and stress responses in mammalian cells.

Because ATF5 is a transcription factor, many questions arise. What are the target genes of ATF5? Does ATF5 regulate the same or a different set of genes as ATF4? Although the physiological role of ATF5 gene regulation in response to amino acid limitation and oxidative stress is not yet understood, ATF5 may be a key transcription factor regulating gene expression with important functions in response to amino acid limitation and oxidative stress in relation to physiological functions and diseases.

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