Interaction of RNA-binding Proteins HuR and AUF1 with the Human ATF3 mRNA 3′-Untranslated Region Regulates Its Amino Acid Limitation-induced Stabilization*

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ATF3 expression is induced in cells exposed to a variety of stress conditions, including nutrient limitation. Here we demonstrated that the mechanism by which the ATF3 mRNA content is increased following amino acid limitation of human HepG2 hepatoma cells is mRNA stabilization. Analysis of ATF3 mRNA turnover revealed that the half-life was increased from about 1 h in control cells to greater than 8 h in the histidine-deprived state, demonstrating mRNA stabilization in response to nutrient deprivation. Treatment of HepG2 cells with thapsigargin, which causes endoplasmic reticulum stress, also increased the half-life of ATF3 mRNA. HuR is an RNA-binding protein that regulates both the stability and cytoplasmic/nuclear localization of mRNA species containing AU-rich elements. Another RNA-binding protein, AUF1, regulates target mRNA molecules by enhancing their decay. Amino acid limitation caused a slightly elevated mRNA level for HuR and AUF1 mRNA. The nuclear HuR protein content was unchanged, and AUF1 protein increased slightly after amino acid limitation, whereas the cytoplasmic levels of both HuR and AUF1 protein increased. Immuno-precipitation of HuR-RNA complexes followed by reverse transcriptionase-PCR analysis showed that HuR interacted with ATF3 mRNA in vivo and that this interaction increased following amino acid limitation. In contrast, the interaction of AUF1 with the ATF3 mRNA is decreased in histidine-deprived cells relative to control cells. Suppression of HuR expression by RNA interference partially blocked the accumulation of ATF3 mRNA following amino acid deprivation. The results demonstrated that coordinated regulation of mRNA stability by HuR and AUF1 proteins contributes to the observed increase in ATF3 expression following amino acid limitation.

Metabolite control of gene expression in mammalian cells is an important aspect of regulating cellular responses to changes in the nutritional status of the organism (1, 2). The availability of amino acids for specific tissues is crucial in the pathology (3, 4) and therapeutic treatment of certain diseases (5). The limitation of amino acids to mammalian cells modulates gene expression at transcriptional (6), post-transcriptional (7–9), and translational (10, 11) levels. The signal transduction pathway related to the regulation of gene expression by amino acid limitation is referred to as the amino acid response. A number of cell stress conditions, including amino acid deprivation (12), ER stress (13), the presence of long double-stranded RNA (14), and heme deficiency (15), leads to increased elf-2α phosphorylation, and consequently, global protein synthesis is repressed. However, under this condition, both transcription (16) and translation (11, 17) of ATF4 are selectively increased, the latter due to short upstream opening reading frames within the ATF4 mRNA. The enhanced production of ATF4 results in the induction of a large number of target genes (18), including asparagine synthetase (ANAS) (16, 19). Another mechanism for increasing mRNA and protein expression following amino acid limitation is mRNA stabilization, which has been documented for the cationic amino acid transporter 1 (cat-1) (9) and the cell cycle regulatory protein p21 (8).

Control of mRNA stability represents a critical regulatory mechanism of gene expression in mammalian cells that is not well understood. It is becoming evident that specific trans-acting factors bind to AU-rich elements (AREs) in the 3′-untranslated regions (UTRs) of a given mRNA and thereby mediate control of mRNA degradation or stabilization. Among the RNA-binding proteins identified so far, HuR and its neuronal homologues HuB, HuC, and HuD have been associated with post-transcriptional stabilization of mRNA for cyclin, vascular endothelial growth factor, tumor necrosis factor-α, c-Myc, interleukins, p21, p27, and p53 (20). In contrast, a number of other RNA-binding proteins, including TTP, BRF1, KSRP, AUF1/hnRNP D, and other members of the hnRNP family of proteins, function as destabilizers of the target mRNA to which they bind. The abundance and subcellular localization of HuR is altered by amino acid limitation, and it stabilizes cat-1 mRNA by binding to the 3′-UTR (9).

ATF3 is a transcription factor for which expression is low in normal and quiescent cells, but can be rapidly induced in response to diverse stress signals, and is likely to be involved in controlling a wide variety of stress-related cellular activities (21). Pan et al. (22) and Jiang et al. (23) demonstrated that the expression of ATF4 is induced in response to amino acid deprivation or to ER stress. Ron and co-workers (18) have shown previously by microarray analysis that in ATF4-deficient cells tunicamycin-induced ATF3 mRNA induction was much less as compared with the wild type. Recently, Wek and co-workers (23) showed that in general control nonderepressible 2-, double-stranded RNA-dependent protein kinase-like endoplasmic reticulum kinase-, and ATF4-deficient cells induction of ATF3 following amino acid limitation is impaired, and they concluded that ATF3 is integral to the stress situation.
Regulation of ATF3 by Amino Acid Limitation

response mediated by the elf-2a kinases. The ATF3 transcript is also known to undergo alternative splicing yielding an extensive set of proteins of different sequence, coding frames, and length (22, 24–26). The longest protein, full-length ATF3, can function as a homodimer, in which case it often acts to repress transcription, or as a heterodimer with other bZIP family members, in which case it can either repress or activate transcription (27). Following amino acid deprivation, there is an increase in multiple ATF3 mRNA species that results from alternative splicing and a change in the ratio among these species (22), suggesting that the splicing machinery is somehow regulated by amino acid availability. However, the net accumulation of ATF3 mRNA molecules could also involve increased stabilization, which is the hypothesis tested in this report.

The experiments in this report establish that mRNA stability contributes to the increased ATF3 expression following amino acid limitation or ER stress and that specific RNA-binding proteins are involved in the regulation of the ATF3 stabilization. Analysis of ATF3 mRNA turnover revealed that the half-life was increased from about 1 h in HepG2 human hepatoma cells maintained in amino acid-complete medium, to greater than 8 h in histidine-deficient medium. ER stress also increased the half-life of ATF3 mRNA, from 1 to 3 h. Immunoprecipitation of HuR-RNA complexes followed by reverse transcriptase-PCR analysis showed that HuR interacts with the ATF3 mRNA in vivo and that the interaction of HuR with ATF3 mRNA increases following amino acid limitation. In contrast, the interaction of AUF1 with the ATF3 mRNA is slightly decreased in histidine-deprived cells relative to control cells. Furthermore, suppression of HuR protein expression using RNAi partially blocked the increased accumulation of ATF3 mRNA resulting from histidine limitation. These results show that HuR is involved in stress-induced ATF3 mRNA stabilization, whereas AUF1 may be a destabilizing factor for this ARE-containing mRNA molecule.

MATERIALS AND METHODS

Cell Culture—Human HepG2 hepatoma cells were cultured in minimal essential medium (MEM, pH 7.4; Mediatech, Herndon, VA), supplemented to contain 10% dialyzed FBS, and 1% nonessential amino acid solution, 4 mM glutamine, 100 µg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 µg/ml amphotericin B, and 10% (v/v) fetal bovine serum (FBS). Cells were maintained at 37 °C in a 5% CO2, 95% air incubator and were replenished with fresh MEM and serum 12 h prior to initiating all treatments. Nutrient deprivation was performed by incubating the cells in complete MEM or MEM lacking histidine (Bio-Rad), as described previously (22). After staining with Fast Green to check for equal loading, the membrane was incubated with 5% blocking solution and two times for 5 min in freshly made TBS/Tween 20% (v/v) Tween 20, and 200 mM NaCl) for 2 h at room temperature with mixing. Immunoblotting was performed using an antibody against HuR or AUF1 at a concentration of 0.8 µg/ml in 5% blocking solution by incubation for 2 h at room temperature (~21 °C). Anti-AUF1 (catalog number sc22368) and anti-HuR (catalog number sc20694) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were washed five times for 5 min in 1× dry milk blocking solution on a shaker and then incubated for 45 min at room temperature with peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a 1:5,000 dilution in 1× dry milk blocking solution. The blots were then washed five times for 5 min in 1× dry milk blocking solution and two times for 5 min in freshly made TBS/Tween (30 mM Tris base, pH 7.5, 0.1% (v/v) Tween 20, and 200 mM NaCl) for 2 h at room temperature with mixing. The bound secondary antibody was detected using an enhanced chemiluminescence kit (Amersham Biosciences) and exposing the blot to Biomax MR film (Eastman Kodak).

RNA Immunoprecipitation Analysis—Immunoprecipitation of protein-RNA complexes was performed by a modified protocol for chromatin immunoprecipitation (29). Briefly, HepG2 cells were seeded at 2.5 × 10^6/150-mm dish with complete MEM and grown for 24 h. Cells were transferred to fresh MEM 12 h before treatment and then incubated in either complete MEM or MEM lacking histidine (MEM-His), each containing 10% dialyzed FBS, for 8 h. After protein-RNA crosslinking with 1% formaldehyde, the cells were lysed with nuclei swelling
buffer (5 mM PIPES, pH 8.0, 85 mM KCl, and 0.5% Nonidet P-40), and the nuclei were pelleted and discarded. Cytoplasmic extract equaling \(-20 \times 10^6\) cells was incubated at 4 °C overnight with 15 μg of either rabbit anti-chicken IgG (nonspecific control) or an antibody against either HuR or AUF1. Cytoplasmic extract not incubated with antibody was saved as an “input” sample. The antibody-bound complex was precipitated by protein A-Sepharose beads (Amersham Biosciences), and the beads were sequentially washed once each for 5 min with low salt, high salt, LiCl, and TE buffers (low salt wash: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl; high salt wash: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl; LiCl wash: 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0; TE buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The protein-RNA complex was eluted from the protein A-Sepharose beads with 250 μl of elution buffer (1% SDS and 0.1 M NaHCO₃) at 37 °C for 15 min. The RNA in the immunoprecipitated complex and the RNA in the previously saved input fraction were released by reversing the cross-linking at 65 °C for 2 h with 200 mM NaCl and 20 μg of proteinase K. The RNA was then extracted from the solution using Trizol reagent (Invitrogen) and subjected to RT-PCR using primers to amplify a sequence in the 3’-untranslated region of the human ATF3 mRNA (sense 5’-GCTGTGACAGGCACGAGCTGTCAGC-3’, antisense 5’-CTGTTCCTCCTCCTTGAGGAGAC-3’).

**RNA Interference Using a Vector-based Short Hairpin siRNA**—An expression plasmid for an siRNA directed against HuR was constructed containing the U6 promoter region from nt \(-265\) to \(+1\), specific for RNA polymerase III, which was amplified by PCR from genomic DNA with the following primers: sense 5’-CGTATGAAATGSGATCCTGACGCGCCCATCTCTCCTTGAGGAGACT-3’; antisense 5’-GCCGATGTCAGGTGCTACACAAAGCGTTTTCCTCCAA-3’. A BsmBI site (underlined) in the antisense primer allowed insertion of siRNA sequences at the first nucleotide of the U6 transcript. The U6 promoter was cloned into the vector pcDNA3.1/zeo and named pcU6K. HuR-specific siRNA oligonucleotides were designed that contained a sense strand (5’-CTACCTCCTTGAAAGATGTCTTGCTGAC-3’) of nucleotide sequence followed by a short spacer (5’-TTCAAGAGATTATTCTTGAGGGAAGT-3’), the reverse complement of the sense strand (5’-CTACCTCCTTGAAAGATGTCTTGCTGAC-3’) and then five thymidines as an RNA polymerase III transcriptional stop signal. The siRNA sequences were synthesized as two complementary DNA oligonucleotides, annealed, and then ligated into the BsmBI site of pcU6K. To ensure the effective depletion of HuR mRNA (more than 70%), two successive transfections with the short hairpin siRNA-containing plasmid were performed. Briefly, HepG2 cells were seeded at 1.0 \(\times 10^6\) per 60-mm dish with complete MEM and grown for 18 h before transfection using CaPO₄ for 12 h (28). Eighteen hours after the first transfection, a second transfection was performed using Superfect for 3 h, as described (22). Total RNA and protein extracts from the transfected HepG2 cells were collected for analysis 24 h after the second transfection.

**RESULTS**

**Effect of Amino Acid Limitation on ATF3 mRNA Synthesis and Turnover**—Although we observed previously that the ATF3 mRNA content of cells is increased after 12 h of amino acid limitation, analysis of the kinetics of mRNA accumulation was necessary to determine whether this increase was transient. Consequently, HepG2 human hepatoma cells were incubated in histidine-free MEM for 0–24 h, and the mRNA level for ATF3 was measured by qPCR (Fig. 1A). An initial increase in ATF3 mRNA content was observed after 2 h of histidine limitation and then reached a maximum of about 15 times the control value at 8 h. Although there was a trend of decline from 8 to 24 h, the level was still elevated by 10 times the control after 24 h.
Regulation of ATF3 by Amino Acid Limitation

To evaluate whether mRNA stabilization contributes to the increase in ATF3 mRNA in HepG2 cells, they were first incubated in MEM without histidine for 12 h to elevate ATF3 mRNA content, and then transferred to either fresh histidine-free MEM or complete MEM, both containing 5 μM ActD (Fig. 1B). ATF3 RNA content was assayed by qPCR, and the rate of decay was analyzed graphically. The half-life was increased from about 1 h in control cells, to greater than 8 h in the histidine-deprived state, documenting a role for mRNA stabilization in response to nutrient deprivation. Treatment of HepG2 cells with thapsigargin (300 nM), which induces an ER stress response through inhibition of the ER Ca\(^{2+}\)-ATPase, also increased the half-life of ATF3 mRNA from 1 to 3 h (Fig. 1C), but the increase was much less than that by amino acid limitation. Analysis of transcription rate from the ATF3 gene revealed that it was only increased by about 2-fold after amino acid limitation (data not shown). These results indicate that amino acid limitation of HepG2 cells elevates the ATF3 mRNA content mainly by increasing mRNA stability.

The 3'-UTR of the ATF3 mRNA Is a Determinant of Stability—Regulation of mRNA stability is often mediated by AU-rich sequences within the 3'-UTR (20). The possible importance of such sequences within the ATF3 3'-UTR was suggested by sequence analysis of the human, chimp, and rat ATF3 mRNA, which revealed that a stretch of 3'-UTR AU-rich regions existed and was highly conserved among all three species. To test whether or not the ATF3 3'-UTR mediates mRNA stability during amino acid limitation, a luciferase reporter construct was made such that a 418-bp fragment (nt 1463–1880) from the ATF3 3'-UTR was inserted between the luciferase coding sequence and the SV40 polyadenylation region in the pGL3-Promoter plasmid (Fig. 2). The half-life of the luciferase mRNA from the pGL3-Promoter plasmid in cells incubated without histidine was increased slightly (Fig. 2A). The addition of the ATF3 3'-UTR to the luciferase mRNA resulted in an increase in mRNA half-life from 2 h in the amino acid complete MEM condition to 8 h in the histidine-free MEM condition (Fig. 2B). This result demonstrates that the ATF3 3'-UTR is capable of conferring amino acid-regulated stabilization to a heterologous mRNA. Furthermore, the fold increase in the half-life of the heterologous mRNA is similar to the increase observed for the endogenous ATF3 mRNA (Fig. 1), indicating that the 418-bp fragment from the ATF3 3'-UTR contains all the sequences required for the regulation of ATF3 mRNA by stabilization.

Effect of Amino Acid Limitation on AUFI and HuR mRNA and Protein Levels—HepG2 cells were incubated in histidine-free MEM for 0–8 h, and the mRNA level for AUFI and HuR was measured by qPCR (Fig. 3). Amino acid limitation caused a slightly elevated mRNA level for both HuR and AUFI mRNA. HuR is a 32-kDa RNA-binding protein predominantly nuclear in localization, but a number of stimuli can cause its translocation to the cytoplasm (30). AUFI or hnRNP D is a member of the heterogeneous nuclear ribonucleoproteins expressed in four isoforms, p37, 40, 42, or 45, through alternative splicing of a common pre-mRNA (31). To establish the kinetics of AUFI or HuR protein content and subcellular localization after amino acid limitation, cytoplasmic extracts or nuclear extracts from control (complete MEM) or histidine-deprived (MEM-His) HepG2 cells were subjected to immunoblotting (Fig. 4). Amino acid limitation increased the cytoplasmic content of both HuR and AUFI (Fig. 4A). For AUFI, the protein was detectable predominantly as the p40/42 isoform. The relative amount of the AUFI isoforms was more equally distributed in the nucleus, and there was a slight increase in abundance at 4–8 h after histidine deprivation (Fig. 4B). In contrast to the cytoplasmic expression, the nuclear protein content for HuR and AUFI was unchanged during amino acid limitation, suggesting that the increase in the cytoplasm may represent new synthesis.

Interaction of RNA-binding Proteins in the Cytoplasm with ATF3 mRNA in Vivo—The ATF3 mRNA has several AU-rich sequences located in its 3'-UTR, but protein binding to the ATF3 mRNA has not been investigated. It has been documented that in C6 rat glioma cells, amino acid deprivation causes HuR to translocate from the nucleus to the cytoplasm and that HuR participates in the amino acid-dependent regulation of the rat cat-1 mRNA content by extending the half-life (9). To test whether or not HuR or AUFI interacts with the ATF3 mRNA in vivo, the ATF3 3'-UTR, but protein binding to the ATF3 mRNA has not been investigated. It has been documented that in C6 rat glioma cells, amino acid deprivation causes HuR to translocate from the nucleus to the cytoplasm and that HuR participates in the amino acid-dependent regulation of the rat cat-1 mRNA content by extending the half-life (9). To test whether or not HuR or AUFI interacts with the ATF3 mRNA in vivo, the 418-bp fragment (nt 1463–1880) of the ATF3 3'-UTR was amplified by PCR and cloned into the XbaI site downstream of the firefly luciferase protein coding sequence in the pGL3-Promoter vector. The control luciferase reporter construct (A) or the luciferase linked to the ATF3 3'-UTR (B) was transfected into HepG2 cells. Twenty four hours after transfection, the cells were incubated in MEM without histidine for 12 h and then transferred to either fresh histidine-free MEM or complete MEM, both containing 5 μM ActD. Total cellular RNA was isolated from the cells at the indicated times, and luciferase mRNA content was assayed by qPCR to analyze the rate of decay.
Regulation of ATF3 by Amino Acid Limitation

Effect on ATF3 mRNA of an Interfering RNA Targeted to the HuR mRNA—If the increased HuR binding to the ATF3 mRNA 3′-UTR following amino acid limitation was responsible for the increased stability, it would be expected that inhibition of HuR expression would blunt the starvation-dependent increase in ATF3 mRNA. RNA interference was performed to specifically knock down HuR expression, through the use of a plasmid-based short hairpin siRNA targeted against a sequence located in the 5′ region of the HuR mRNA. The negative control siRNA was a scrambled sequence. Total RNA and cell extracts from siRNA-transfected HepG2 cells were analyzed by qPCR to detect the level of HuR, ASNS, ATF4, p21, or ATF3 mRNA and by immunoblot analysis to detect HuR protein (Fig. 6). Twenty four hours after transfection with the HuR siRNA, the HuR mRNA level was decreased by ~60% in the MEM condition and ~80% in cells maintained in MEM without histidine for 8 h, relative to the control siRNA-transfected cells (Fig. 6A). Under these conditions, cytoplasmic HuR protein was reduced to 20–30% of the control for both MEM and MEM-His-treated cells (Fig. 6A). Reducing the HuR protein level had little effect on the induction of ASNS or ATF4 mRNA following amino acid limitation (Fig. 6B), consistent with the lack of a contribution of mRNA stabilization to the increase in ASNS (19) or ATF4 (16) mRNA. However, the increase in p21 mRNA following amino acid limitation is almost entirely dependent on mRNA stabilization (8), and HuR binding to the p21 mRNA is not affected by amino acid limitation (36).

vivo, HepG2 cells were treated with formaldehyde to cross-link protein to RNA, and total cellular extracts were then incubated with either an anti-HuR, anti-AUF1, or a nonspecific antibody (rabbit anti-chicken IgG). The immunoprecipitated RNA was isolated, and specific sequences for ATF3 mRNA were amplified by PCR (Fig. 5). In an initial test, a primer set that amplifies a 70-bp fragment of the ATF3 mRNA 3′-UTR was used to examine the specificity of the method, and gel electrophoresis of the PCR showed a single product as expected (Fig. 5A). As a negative control, there was much less precipitation of the ATF3 mRNA with the nonspecific antibody (Fig. 5A, n/s IgG). Quantitative assessment of RNA protein binding was performed by qPCR analysis of the precipitated RNA using primers specific for ATF3 (Fig. 5, A and C). RNA isolated after cross-linking, but before immunoprecipitation (input), was used as a positive control sample. There was no difference in binding between the control and the histidine-deprived conditions for the negative control antibody (data not shown), so the AUF1 and HuR data were normalized to these nonspecific IgG values. At each time point tested, there was increased HuR binding to the ATF3 mRNA in amino acid-deprived HepG2 cells, compared with control cells (Fig. 5B). At the same time, the interaction of AUF1 with the ATF3 mRNA transiently increased (2 h). At 4 and 8 h the AUF1 binding in both the control and the amino acid-deprived cells increased, but the increase in the controls was modest compared with the amino acid-deprived cells, such that there was a relative decline in the AUF1 binding following histidine removal from the medium (Fig. 5C). These data are consistent with the hypothesis that HuR and AUF1 play opposite roles (stabilizing and destabilizing, respectively) in regulating mRNA stability (30).
mRNA has been documented (32). Extending those observations, the data showed that the nutrient-regulated increase in p21 mRNA was completely blocked in cells transfected with the HuR siRNA (Fig. 6B). Thus, ASNS or ATF4 and p21 serve as negative and positive controls, respectively, for amino acid-dependent stabilization and HuR action. The increase in the level of ATF3 mRNA in the histidine-deprived cells was blocked by more than 50% (Fig. 6B). These results document that the increased association of HuR protein with the ATF3 mRNA extends its half-life, and together with the earlier work of Yaman et al. (9) on cat-1 stabilization, the results indicate that HuR is an important component of the amino acid response pathway.

**DISCUSSION**

ATF3 is a member of the ATF/CREB subfamily of the larger basic region/leucine zipper (bZIP) transcription factor family, which also includes members of the CCAAT/enhancer-binding protein (C/EBP) family and Jun/Fos. Normally expressed at low levels in cells, ATF3 expression is rapidly induced in response to diverse stress signals and is likely to be involved in controlling a number of stress-related responses, including nutrient stress (22–24). For example, ATF3 is expressed in the islets of mice that have developed insulin or diabetes and also in human patients with type 1 or type 2 diabetes (33). Those authors also showed that ATF3 is a regulator of stress-induced pancreatic β-cell apoptosis.

The data in this report reveal an important molecular mechanism that contributes to the ATF3 mRNA induction following amino acid deprivation, and the results illustrate the following novel observations. 1) Analysis of ATF3 mRNA turnover revealed that mRNA stabilization contributes to the amino acid-dependent or ER stress-induced increase in ATF3 expression. 2) The ATF3 3′-UTR sequence is capable of conferring amino acid-regulated mRNA stability to a heterologous mRNA. 3) Amino acid limitation caused a slightly elevated mRNA level for both HuR and AUF1. 4) The nuclear protein content for AUF1 was unchanged, but the cytoplasmic content of the AUF1 p40/42 isoforms increased during amino acid limitation. 5) Immunoprecipitation of HuR-RNA complexes followed by PCR analysis showed that HuR interacts with the ATF3 mRNA in vivo and that the HuR-ATF3 mRNA complex increases in abundance following amino acid limitation. However, after 4–8 h of histidine deprivation, AUF1 binding to the ATF3 mRNA was lower in the histidine-deprived cells. 6) A reduction of HuR expression using RNAi blocked the increased accumulation of ATF3 mRNA by more than 50%, consistent with a contribution of both transcription and stabilization to the increase in ATF3 mRNA content following amino acid limitation.

The regulated degradation/stabilization of many mRNAs is dependent upon AU-rich elements (ARE) present within their 3′-UTR. Typically, mRNA degradation starts with deadenylation of the poly(A) tail, and the ARE-binding proteins are involved in controlling that process as well as helping to recruit the exosome, a multiprotein complex of
RNases involved in rapid 3’-to-5’ degradation of the mRNA (34). Given that different signal transduction pathways selectively stabilize ARE-containing mRNAs, the ARE-binding proteins are likely targets of the pathways. For example, the c-Jun NH₂-terminal kinase and phosphatidylinositol 3-kinase pathways are involved in AUUUA-mediated interleukin-2 and -3 mRNA turnover (35, 36), and activation of p38 mitogen-activated protein kinase induces stabilization of a number of ARE-containing mRNAs (37, 38). With regard to ATF3, the only previous report on mRNA stability involved the protein synthesis inhibitor anisomycin. At anisomycin concentrations so low that there is no inhibition of translation, the drug still caused activation of the c-Jun NH₂-terminal kinase signal transduction pathway and an increase in the steadystate level of ATF3 mRNA (39). This increase was the result of both increased mRNA synthesis and a modest increase in stability of the ATF3 mRNA (t½ of about 90 min compared with <60 min for the control) (39). In this report, we demonstrate that the increased expression of ATF3 following amino acid limitation or ER stress of HepG2 cells involves increased stability of the ATF3 mRNA, such that the half-life is extended by 8-fold following histidine limitation.

A number of members of the hnRNP family of proteins, including HuR and AUF1, have been identified to interact with AREs and affect the stability of ARE-containing transcripts (40). HuR is a member of the human embryonic lethal abnormal vision family of RNA-binding proteins, ubiquitously expressed in all cell types. HuR plays a role in the nuclear export of mRNA, in control of translation, and in the stabilization of several ARE-containing mRNAs (20). It has been documented that HuR and AUF1 can bind to a common target mRNA, in a competitive fashion, in the form of stable nuclear RNP complexes (30). However, in the cytoplasm, an AUF1-bound mRNA undergoes exosome-mediated decay, whereas an HuR-bound mRNA is stabilized and is associated with the translation machinery. Yaman et al. (9) previously demonstrated in RNA gel shift experiments that HuR could bind in vitro to the ARE within the 3’-UTR sequence of the cat-1 mRNA. They also observed that amino acid limitation caused HuR accumulation in the cytoplasm of C6 rat glioma cells and that the cytoplasmic HuR binding to the cat-1 ARE showed a transient increase during amino acid deprivation. In this report, we present further evidence for the critical role that RNA destabilization/stabilization plays in the amino acid response, and we provide in vivo evidence for an interaction between the ATF3 mRNA and the ARE-binding proteins HuR and AUF1.

The 3’ region of the ATF3 mRNA contains several AUUUA sequences, which may function as stabilizing elements. There are examples of mRNA studies in which these AUUUA motifs are mutated, and yet the mRNA is still subject to regulation by destabilization/stabilization (41). Therefore, it has been suggested that the sequence requirements for mRNA stability regulation are more flexible and, consequently, more complex than initially assumed. Further research will be necessary to identify the specific HuR-binding sites in the ATF3 mRNA and then to establish which of those sites contributes to the amino acid responsiveness. However, the present results demonstrate that HuR binds the ATF3 mRNA in vivo through RNA immunoprecipitation of HuR RNA complexes from cytoplasmic lysates of fed and amino acid-deprived HepG2 cells. The increase in ATF3 mRNA–HuR complexes in histidine-deprived HepG2 cells is consistent with the half-life increase of ATF3 mRNA in vivo. Although HuR is expressed in both fed and amino acid-deprived cells, HuR protein content is increased in the cytoplasm in response to amino acid signaling in HepG2 cells, as reported previously for C6 cells (9). AUF1 overexpression in various cell lines has been found to accelerate ARE-dependent mRNA decay (42) and selective knockdown of the AUF1 isoforms by siRNA stabilized mRNA (43). Consistent with its role as a destabilizer, when ATF3 mRNA increase in amino acid-deprived HepG2 cells, AUF1 binding to ATF3 mRNA decreased. More importantly, these changes in the cytoplasmic interaction of HuR and AUF1 with ATF3 mRNA parallel the time course of ATF3 mRNA accumulation in vivo, and suppression of HuR by RNAi partially inhibited ATF3 mRNA accumulation induced by amino acid deprivation (29).

Given that HuR stabilizes only a subset of ARE-containing mRNAs (44), it remains to be established whether the role of HuR in amino acid deprivation-mediated mRNA stabilization of the cat-1 (9), p21, and ATF3 mRNA will be the general rule for mRNA molecules for which turnover is regulated by amino acid control. The observation of amino acid-regulated stabilization of ATF3 mRNA in this report provides further evidence for this mechanism in the expression of critical stress-sensing factors such as ATF3 and p21 following amino acid limitation. It has been reported recently that components involved in micro-RNA processing and function, such as Dicer1 and Argonaute1 and 2, are required for the decay of ARE-containing mRNA such as tumor necrosis factor-α in both Drosophila S2 cells and HeLa cells (45). A possible link between the effect of micro-RNA molecules on mRNA instability and amino acid availability may reveal novel regulatory mechanisms that cells use to regulate gene expression in response to nutrient stress.

Acknowledgments—We thank other members of the laboratory for technical advice, reagents, and helpful discussion.

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J. Biol. Chem. 2005, 280:34609-34616.
doi: 10.1074/jbc.M507802200 originally published online August 17, 2005

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