Amino Acid Deprivation and Endoplasmic Reticulum Stress Induce Expression of Multiple Activating Transcription Factor-3 mRNA Species That, When Overexpressed in HepG2 Cells, Modulate Transcription by the Human Asparagine Synthetase Promoter*

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Transcription from the ASNS (asparagine synthetase) gene is increased in response to either amino acid (amino acid response) or glucose (endoplasmic reticulum stress response) deprivation. These two independent pathways converge on the same set of genomic cis-elements within the ASNS promoter, referred to as nutrient-sensing response element-1 and -2. Chromatin immunoprecipitation analysis provides the first in vivo evidence for activating transcription factor (ATF)-3 binding to the proximal ASNS promoter containing the nutrient-sensing response element-1 sequence. Overexpression of the full-length ATF3 protein caused a concentration-dependent biphasic response in ASNS promoter-driven transcription. Both amino acid limitation and activation of the endoplasmic reticulum stress response by glucose deprivation caused an increase in ATF3 mRNA content. However, reverse transcriptase-PCR analysis revealed that the increase in the ATF3 mRNA species detected by Northern analysis actually encoded both full-length ATF3 and two predicted truncated ATF3 isoforms (ATF3ΔZip2e and ATF3ΔZip3). Based on sequence analysis, one of the predicted truncated proteins (ATF3ΔZip3) is likely incapable of binding DNA; and yet, exogenous expression of the cDNA enhanced starvation-induced or ATF4-activated ASNS transcription, possibly by sequestering corepressor proteins. Collectively, the results provide evidence for a potential role of multiple predicted ATF3 isoforms in the transcriptional regulation of the ASNS gene in response to nutrient deprivation.

ASNS (asparagine synthetase) encodes an enzyme that catalyzes the glutamine- and ATP-dependent conversion of aspartic acid to asparagine (1). ASNS expression in mammalian tissues is highly regulated by the nutritional status of the cell. The activation of either the amino acid response pathway by amino acid deprivation or the endoplasmic reticulum stress response (ERSR) pathway, also known in yeast as the unfolded protein response, converges on the same set of genomic cis-elements within the ASNS promoter and leads to the accumulation of ASNS mRNA (2). Mammalian cells have evolved complex responses to cell stress, and these responses often serve as a protective function. The up-regulation of ASNS transcription upon nutrient stress suggests that ASNS is a critical cellular defense gene, although the exact roles of ASNS and the molecular mechanisms for activation have not been extensively investigated. Therefore, characterization of the transcriptional regulation of the ASNS gene following amino acid deprivation will not only provide information on the cellular mechanisms for reacting to amino acid limitation (3–5), but may also reveal insight into the importance of ASNS in maintaining cellular function.

Analysis of the proximal promoter of the human ASNS gene by Guerrini et al. (6) identified a palindromic sequence (5’-CATGATG-3’) at nucleotides −70 to −64 within the human ASNS promoter that functions as an amino acid response element. Using mutational analysis, electrophoresis mobility shift assay, and in vivo footprinting experiments, Barbosa-Tessmann et al. (7, 8) demonstrated that two cis-elements termed nutrient-sensing response element-1 (NSRE-1) (5’-TGTAGAAC-3’, nucleotides −68 to −60) and NSRE-2 (5’-GTGTCAGAC-3’, nucleotides −48 to −43) in the ASNS promoter sequence are essential for transcriptional activation by both the amino acid response and ERSSR pathways. Together, NSRE-1 and NSRE-2 function as an enhancer in that transcriptional activity is orientation- and location-independent, and they can transfer amino acid responsiveness to a heterologous promoter (9). In vitro binding experiments and overexpression of specific transcription factors and their dominant-negative mutants have demonstrated that activation of the ASNS gene involves activating transcription factor (ATF)-4 (10) and CCAAT/enhancer-binding protein (C/EBP)β binding to the NSRE-1 site (11).

This study was designed to determine whether ATF3 contributes to the nutrient-dependent regulation of ASNS expression. ATF3 is expressed at low levels in normal and quiescent cells, but can be rapidly induced in response to diverse extracellular stress signals and might be involved in controlling a wide variety of cellular activities (12–14). Alternative splicing within the ATF3 gene is known to occur, and truncated ATF3 isoforms have been reported (14, 15). In contrast to full-length ATF3 (ATF3–FL), which forms homodimers and, in this way, functions as a transcriptional repressor (13), the truncated isoforms (ATF3ΔZip, ATF3ΔZip2a, and ATF3ΔZip2b) can function transactivators; ChIP, chromatin immunoprecipitation; MEM, minimal essential medium; FBS, fetal bovine serum; ER, endoplasmic reticulum.
ATF3 Modulates the Human ASNS Gene

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Our data from HepG2 hepatoma cells show that ATF3 mRNA content is increased by either amino acid or glucose deprivation, but that the induced mRNA contains multiple sequences that arise from alternative splicing. When translated, these mRNA species would encode multiple ATF3 isoforms, including two newly identified truncated proteins (ATF3 ΔZip2c and ATF3 ΔZip3). Chromatin immunoprecipitation (ChIP) experiments established that ATF3-FL bound to the proximal ASNS promoter containing the NSRE-1 sequence in vivo. Overexpression of ATF3-FL caused a biphasic response. Low plasmid concentrations resulted in a further enhancement of transcription from the ASNS promoter mediated by ATF4, but high plasmid concentrations caused an antagonism of the action of ATF4. Expression of the ATF3 ΔZip2c cDNA in HepG2 cells had no detectable effect on ASNS promoter-driven transcription. In contrast, transfection of the cDNA for the other newly identified variant, ATF3 ΔZip3, enhanced starvation-induced transcription mediated by ATF4 in a concentration-dependent manner.

MATERIALS AND METHODS

Cell Culture—HepG2 hepatoma cells were cultured in minimal essential medium (MEM; Mediatech, Herndon, VA) (pH 7.4) supplemented to contain the recommended amount of the manufacturer’s nonessential amino acid solution, 4 mM glutamine, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 μg/ml amphotericin B, and 5% (v/v) fetal bovine serum (FBS). Cells were maintained at 37 °C in a 5% CO2 and 95% air incubator. Given that ASNS promoter expression is induced by nutrient deprivation, cell cultures were replenished with fresh MEM and serum 12 h prior to initiating all treatments. Nutrient deprivation was performed by incubating the cells for 12 h in complete MEM, in MEM lacking histidine or glucose, or in MEM containing 5 μg/ml tunicamycin or 300 μM thapsigargin. All treatment media contained 5% dialyzed FBS during the 12-h treatment.

Northern Analysis—HepG2 cells (2.65 × 106 cells) were seeded on 60-mm dishes for 24 h; given fresh MEM for an additional 12 h before treatment; and then transfected to fresh complete MEM, glucose-free MEM, or histidine-free MEM for 12 h, each supplemented with 5% dialyzed FBS. To address the role of transcription or translation during amino acid starvation, actinomycin (5 μM) or cycloheximide (100 μM) was added to parallel MEM. Total cellular RNA was isolated using an RNaseasy mini kit (QIAGEN Inc., Valencia, CA) following the supplier’s recommendations. For Northern analysis, 15 μg of RNA was size-fractionated and capillary-transferred to a Hybond-N nylon membrane. Following hybridization and washing, the blots were exposed to X-ray film (Fuji Medical Systems USA) and then quantified with a densitometer.

Identification of Multiple ATF3 mRNA Species by RT-PCR—HepG2 cells (2.65 × 106 cells) were seeded on 60-mm dishes for 24 h; given fresh MEM for an additional 12 h; and then transfected for 12 h to fresh complete MEM or to histidine-free MEM, each supplemented with 5% dialyzed FBS. Cytoplasmic RNA was extracted using Concert™ cytoplasmic RNA reagent (Invitrogen) to eliminate DNA and RNA contaminants according to the procedure recommended by the supplier. ATF3 sequences were amplified by the Qiagen one-step RT-PCR kit. The RT reaction was performed at 50 °C for 30 min, followed by 95 °C for 15 min, to activate HotStarTaq DNA polymerase and to inactivate the RT. For ATF3-FL, PCR was performed at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s for 30 cycles. In contrast, the amplification temperature for the alternatively spliced mRNA species was 60 °C. The primers used to amplify ATF3-FL were the 5′-primer (5′-ATGATGCTTCAACACCCAGGC-3′) and the 3′-primer (5′-TATGCTCTGGATGTCCCTTC-3′). The primers used to amplify ATF3 ΔZip2c and ATF3 ΔZip3 mRNA species were the 5′-primer (5′-ATGATGCTTCAACCCAGGC-3′) and the Zip3 primer (5′-CCTCACTCCAAAGG-3′), as originally chosen by Hashimoto et al. (14). The PCR amplification products were subcloned into the TOPO-TA cloning vector (Invitrogen) for sequencing.

To measure the relative amount of the three different ATF3 mRNA species, quantitative real-time PCR analysis was performed using a DNA Engine Opticon 2 system (MJ Research, Inc., Reno, NV) and detection by SYBR green I. The reactions were incubated at 50 °C for 30 min, followed by 95 °C for 15 min to activate the polymerase and amplification at 95 °C for 15 s and 60 °C for 30 s for 35 cycles. After PCR, melting curves were acquired by stepwise increases in the temperature from 55 to 95 °C to ensure that a single product was amplified in each reaction. Samples from at least three independent time courses were analyzed.

Transient Transfection and Expression—The rat ATF4 cDNA, generously provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA) (17), was cloned into the pcDNA3.1/myc-his(-) vector (Invitrogen), and its expression was driven by the cytomegalovirus promoter. All ATF3 cDNAs used for expression studies were subcloned into pcDNA3.1/zeo (Invitrogen) to generate the ATF3 expression vectors driven by the cytomegalovirus promoter. The ASNS-175V-51 promoter/Luc reporter plasmid was made by inserting nucleotides −173 to +51 of the ASNS promoter upstream of the firefly luciferase reporter gene using the HindIII site of the pGL3 plasmid (Promega, Madison, WI). ASNS-175V-51 plasmids were seeded on 2 × 106 cells/60-mm dish, before transfection with Superfect reagent (QIAGEN Inc.) at a ratio of 6 μl of Superfect to 1 μg of DNA. For each transfection, 0.5 μg of the ASNS-175V-51 promoter/Luc plasmid was used along with the indicated amounts of the transcription factor expression plasmids. The total amount of transfected DNA was kept constant among experimental groups by the addition of empty pcDNA3.1 plasmid. Eighteen hours following transfection, the cells were transfected to fresh complete MEM containing 5% dialyzed FBS for 12 h before preparing cell extracts for analysis of luciferase activity. To prepare the extracts, cells were washed three times with phosphate-buffered saline and treated with 100 μM of passive lysis buffer (Promega). The lysates were collected and stored at −80 °C until used. Twenty microliters of each extract was used for firefly luciferase assays using the luciferase reporter assay system (Promega), and luminescence was measured with a luminescence reader. The luciferase activity measured in each assay was normalized to the protein concentration in the corresponding cell lysate. At least three independent transfections were performed for each experimental condition, and all experiments were repeated with separate batches of cells.

ChIP Analysis—ChIP analysis was performed following the protocol of Upstate Biotechnology, Inc. (Lake Placid, NY) with minor modifications. Briefly, cells were seeded at 2 × 106/150-mm dish, grown for 24 h, transferred to fresh MEM for 12 h, and then treated either in complete MEM or in MEM lacking histidine for 5 h. Protein-DNA cross-linking was initiated by adding formaldehyde directly to the culture medium to a final concentration of 1% and stopped 10 min later by adding 2 M glycine to a final concentration of 0.125 M. Sonication was performed using a Sonic Dismembrator (Fisher) for five bursts at power 9 for 40 s with 2 min cooling on ice between each burst. Extract equalizing 1 × 107 cells or 72 μg of DNA was incubated with 10 μl of an antibody against the C terminus of ATF3 (Santa Cruz Biotechnology), and then the antibody-bound complex was precipitated by protein A-Sepharose beads (Amersham Biosciences). The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking at 65 °C for 4 h and purified using a QIAquick PCR purification kit (QIAGEN). DNA analysis was performed using quantitative real-time PCR using primers designed by Vector NTI Version 7.1 software (InforMax Inc., Frederick, MD) to amplify ASNS genomic sequence −87 to −22 (human ASNS-87V primer, 5′-TGTTTGGTCTCCGGACCAT-3′; and human ASNS-22R primer, 5′-GTGGTGCTCCTGGAGCAAC-3′).
primer, 5′-CGCTTATAACCGACCTGGCTCCT-3′). Quantitative real-time PCR analysis was performed using the DNA Engine Opticon 2 system and detection with SYBR green I. The PCRs were set up in a reaction volume of 25 µl. 2-Fold serial dilutions of input chromatin were used to generate a standard curve for determining the relative amount of products. The standards and the samples in duplicate were simultaneously used to generate a standard curve for determining the relative amount over the control sample. Samples from at least three independent transfections were performed for each experiment, and the data are reported as the means ± S.D. RNA was isolated and subjected to Northern analysis (20 µg of RNA/lane) for ATF3 content or for ribosomal protein L7a mRNA content as a negative control. The gel in B shows a typical autoradiographic result, and the graph represents the mean ± S.D. of four independent RNA preparations. Total cell extracts were subjected to immunoblotting (C) as described under “Materials and Methods.”

RESULTS

Two separate cis-elements, NSRE-1 and NSRE-2, are both required for induction of the ASNS gene in response to activation of the amino acid response or ER stress pathway (8). The NSRE-1 sequence has been shown to bind both ATF4 and C/EBPβ (10, 11). During the course of the previous studies, unpublished electrophoresis mobility shift assay experiments revealed a small but reproducible amount of ATF3 binding to the NSRE-1 complex. The experiments described below extend those initial observations and investigate the regulatory role of ATF3 in ASNS gene expression.

ATF3-FL Modulates ASNS Promoter Activity—To determine whether ATF3-FL expression alters ASNS promoter activity, HepG2 cells were transiently cotransfected with the ASNS−173/+51 promoter/Luc reporter plasmid and 0.5 µg of an ATF3-FL expression plasmid. After 36 h, the cells were transferred to complete MEM or to MEM lacking either histidine or glucose for 12 h prior to measurement of luciferase activity for ASNS activity (A), Northern analysis for ATF3 mRNA (B), or immunoblotting for ATF3-FL protein (C). The luciferase activity measured in each assay was normalized to the protein concentration in the cell lysate. At least three independent transfections were performed for each experiment, and the data are reported as the means ± S.D. RNA was isolated and subjected to Northern analysis (20 µg of RNA/lane) for ATF3 content or for ribosomal protein L7a mRNA content as a negative control. The gel in B shows a typical autoradiographic result, and the graph represents the mean ± S.D. of four independent RNA preparations. Total cell extracts were subjected to immunoblotting (C) as described under “Materials and Methods.”

Fig. 1. ATF3-FL represses ASNS promoter activity, and ATF3 mRNA content is elevated by amino acid or glucose deprivation. HepG2 cells grown in 24-well cluster trays (2 × 105 cells/well) were transiently cotransfected with 0.5 µg of the ASNS−173/+51 promoter/Luc reporter plasmid and 0.5 µg of the ATF3-FL expression plasmid as described under “Materials and Methods.” The control cells were transfected with empty pcDNA3.1 plasmid instead of ATF3-FL-containing vector. After 36 h, the cells were transferred to complete MEM or to MEM lacking either histidine or glucose for 12 h prior to measurement of luciferase activity for ASNS activity (A), Northern analysis for ATF3 mRNA (B), or immunoblotting for ATF3-FL protein (C). The luciferase activity measured in each assay was normalized to the protein concentration in the cell lysate. At least three independent transfections were performed for each experiment, and the data are reported as the means ± S.D. RNA was isolated and subjected to Northern analysis (20 µg of RNA/lane) for ATF3 content or for ribosomal protein L7a mRNA content as a negative control. The gel in B shows a typical autoradiographic result, and the graph represents the mean ± S.D. of four independent RNA preparations. Total cell extracts were subjected to immunoblotting (C) as described under “Materials and Methods.”
that the ATF3 gene is activated by a variety of stress responses (13).

To test the role of de novo RNA synthesis in the ATF3 mRNA increase, HepG2 cells were histidine-deprived in the presence or absence of 5 μg/ml actinomycin D, and then total RNA was analyzed for ATF3 mRNA content, with ASNS mRNA serving as a positive control (Fig. 3). The ATF3 mRNA content was detectably increased within 1 h of histidine depletion and reached a maximum value of ~16 times the control (MEM) at 6 h (Fig. 3B). Consistent with a possible regulatory role of ATF3 in the ASNS gene, the ASNS mRNA content lagged behind the ATF3 mRNA content and was not increased until 3 h after removing histidine from the medium, reaching a value of ~16 times the control at 12 h (Fig. 3C). Inhibition of de novo RNA synthesis by actinomycin D completely prevented the increase in ATF3 mRNA accumulation in response to amino acid limitation, indicative of transcriptional control by stress stimuli (13). ASNS mRNA accumulation was also completely prevented by actinomycin D, consistent with its known transcriptional regulation by amino acid limitation (2). If ATF3 represents an upstream step in ASNS gene regulation, it is of mechanistic interest to determine whether the nutrient deprivation-induced expression of ATF3 mRNA requires newly synthesized proteins. The increase in ATF3 mRNA in response to amino acid limitation was completely insensitive to blocking protein synthesis by cycloheximide, suggesting that the necessary regulatory proteins were present prior to nutrient deprivation (data not shown).

Identification and Analysis of Predicted ATF3 Isoforms—As described in the Introduction, certain cell types express truncated ATF3 isoforms (14, 15). To provide the first analysis of these isoforms in human HepG2 hepatoma cells and to further characterize the expression of ATF3 during amino acid limitation, we analyzed HepG2 RNA by RT-PCR using the oligonucleotide primer sequences originally described by Hashimoto et al. (14) to identify truncated ATF3 mRNA species (Fig. 4A). RNA from HepG2 cells incubated in MEM or in MEM lacking histidine for 12 h was used to amplify the ATF3-FL coding region (Fig. 4B). A single band of the expected length was detected, and the amount of this ATF3-FL mRNA was increased significantly by histidine deprivation. Alternatively, if primers were used to test for the presence of ATF3ΔZip (14), two bands of ~500 and 1400 bp were observed (Fig. 4C). Interestingly, both bands were different from the predicted size of 554 bp based on previously published reports for ATF3ΔZip (14, 15). Both of the newly identified products were increased in amount by histidine deprivation (Fig. 4C), and sequencing indicated that both are alternatively spliced forms of ATF3, neither of which has been reported previously. To be consistent with the nomenclature used for the other ATF3 variants, based on their sequences and predicted protein structures, the newly identified ATF3 clones were designated ATF3ΔZip2c (~500 bp) and ATF3ΔZip3 (~1400 bp). A quantitative measurement in-

Fig. 2. Time course of ATF3 mRNA content following ER stress. HepG2 cells were incubated for 0–18 h in MEM, in MEM containing tunicamycin (Tm; 5 μg/ml) or thapsigargin (Tg; 300 nm), or in MEM lacking glucose. At the times indicated, RNA was isolated and subjected to Northern analysis (20 μg of RNA/lane) for ATF3 or ribosomal protein L7a mRNA content. The data were quantified and are expressed as a ratio of the value obtained for ribosomal protein L7a. The results shown in the gel represent a typical blot, and the graph illustrates the means ± S.D. of three independent experiments. Where not shown, the error bars are contained with the symbol.

Fig. 3. Induction of ATF3 and ASNS mRNA content by histidine deprivation requires de novo RNA synthesis. HepG2 cells were incubated for 0–12 h in MEM or in MEM lacking histidine in the presence or absence of 5 μg/ml actinomycin D (ActD). At the times indicated, RNA was isolated and subjected to Northern analysis (20 μg of RNA/lane) for ATF3, ASNS, or ribosomal protein L7a mRNA content. The data were quantified and are expressed as a ratio of the value obtained for ribosomal protein L7a. The results shown in A represent a typical blot, and the graphs for ATF3 (B) and ASNS (C) illustrate the means ± S.D. of three independent experiments.
dicated that the relative amount of either ATF3/H9004 Zip2c or ATF3/H9004 Zip3 is only a small fraction of that of ATF3-FL mRNA (Fig. 4D). However, the relative increase in the ATF3ΔZip3 mRNA was much greater than that in either ATF3/H9004 Zip2c or ATF3-FL (Fig. 4E). This result suggests that the increase in ATF3/H9004 Zip3 synthesis may be a specific response to amino acid deprivation.

Alignment of the nucleotide sequences showed that ATF3ΔZip2c arose from a previously unrecognized consensus 5’-donor and 3’-acceptor splice site within exon B that results in the loss of 87 bases from exon B and therefore creates two exons designated as exons B1 and B2 (Fig. 5, A and B). Sequencing also showed that ATF3ΔZip2c retains both exons D and D’, as is the case for ATF3ΔZip2a and ATF3ΔZip2b (15), so the predicted ATF3ΔZip2c protein is composed of 106 amino acids and would differ from the ATF3ΔZip2a and ATF3ΔZip2b proteins by the deletion of 29 amino acids near the N terminus (Fig. 5, B and C). As a result, the putative ATF3ΔZip2c protein would have a shorter activation domain compared with the ATF3ΔZip2a and ΔZip2b isoforms; but like these two isoforms, ATF3ΔZip2c would also have a C-terminal truncation that deletes much of the leucine zipper dimerization domain. The newly identified ATF3ΔZip3 mRNA has exon B intact, as in ATF3-FL; but in the case of ATF3ΔZip3, no splicing occurs between exons C and D’ (Fig. 5A). Therefore, the ATF3ΔZip3 mRNA has an insertion extending from exons C to D’ that is designated exon C’ (Fig. 5, A and B). This additional mRNA sequence encodes a termination codon resulting in a truncated protein of 120 amino acids and a nearly complete loss of the leucine zipper dimerization domain (Fig. 5C).

Neither of these isoforms was reported by Chen et al. (15) in HeLa cells. The reason for this difference is not known, but
Fig. 5. Schematic representation of the ATF3 gene and the mRNA species generated by alternative splicing. The genomic arrangement of all known splicing isoforms is compared with that of ATF3-FL in A. Exons A–E and C–E' are indicated by boxes, and the single translation start site (ATG) and the translation stop codons (TAA, TAG, and TGA) for each isoform are shown. The details for variation in splicing of exons B, C, and D' are discussed under “Results.” The splice variants that generate ATF3ΔZip2c and ATF3ΔZip3 are shown in B. The predicted protein structure for each of the ATF3 mRNAs is shown in C, with the activation (Act), repression (Rep), basic, and leucine zipper regions designated. AA, amino acids.
both tissue-type and regulatory factors may play a role in determining which of these splicing events occurs. Using primers from exons B and E, only the ATF3-FL mRNA was detected, indicating that neither ATF3ΔZip2c nor ATF3ΔZip3 mRNA contains exon E (data not shown). To test whether or not these differentially spliced variants are unique to the transformed HepG2 hepatoma cells, the same oligonucleotide primers for RT-PCR amplification were used with RNA from primary cultured human hepatocytes incubated with or without amino acids, and the same mRNA species were obtained (data not shown).

Regulation of ASNS Promoter Activity by ATF3-FL and ATF4—HepG2 cells were cotransfected with the ASNS promoter/Luc reporter plasmid along with an ATF3-FL expression vector and/or the ATF4 expression vector (Fig. 6). When HepG2 cells were transfected with ATF3-FL alone at concentrations ranging from 0.1 to 200 ng of plasmid DNA, ASNS-driven transcription was enhanced in a concentration-dependent manner, with a maximum induction of ~30 times the MEM control value. Conversely, transfection of HepG2 cells with ATF3-FL at concentrations from 1 to 500 ng resulted in a concentration-dependent repression of the basal promoter activity. However, when HepG2 cells were cotransfected with ATF3-FL and ATF4 together, a biphasic response was observed (Fig. 6). When cells were cotransfected with the ATF4 expression plasmid at levels below 10 ng, further induction of ASNS promoter activity was observed at the lowest level of ATF3-FL plasmid, suggesting transcriptional stimulation; but as the level of ATF3-FL was increased, repression was observed. However, when the ATF4 plasmid level was 100 ng, coexpression of ATF3-FL at 10–1000 ng produced only repression of ASNS promoter activity.

Regulation of ASNS Promoter Activity by Transfection of ATF3ΔZip2c and ATF3ΔZip3—Given the apparent increased expression of the alternative ATF3 mRNAs following amino acid limitation, their potential roles in regulating ASNS expression was investigated. HepG2 cells were cotransfected with the ASNS promoter/Luc reporter plasmid along with an ATF3ΔZip2c expression vector and/or the ATF4 expression vector. As expected, HepG2 cells transfected with ATF4 alone exhibited a concentration-dependent induction of luciferase activity (Fig. 7). In contrast to ATF3-FL, transfection of ATF3ΔZip2c alone had no effect on the basal transcription rate; and when cotransfected with ATF4, ATF3ΔZip2c showed no repression or activation of ATF4-induced promoter activity. The results suggest that, if synthesized, the ATF3ΔZip2c protein does not regulate ASNS promoter activity under these experimental conditions. This lack of activity may be the result of the truncated activation domain of ATF3ΔZip2c relative to the other isoforms that have complete activation domains (Fig. 5C). Unfortunately, no antibody is commercially available to test for synthesis of the putative truncated proteins.

Experiments were also performed to test whether or not transfection of the ATF3ΔZip3 cDNA affects ASNS-driven transcription (Fig. 8). HepG2 cells transfected with 0.01–10 ng of ATF3ΔZip3 vector alone showed a slight trend toward higher rates of transcription. However, when cells were cotransfected with combinations of ATF3ΔZip3 and ATF4, a synergistic activating effect of ATF3ΔZip3 on ATF4 action was observed (Fig. 8). At each concentration of ATF4 plasmid from 0.1 to 10 ng, the simultaneous addition of ATF3ΔZip3 caused a concentration dependent enhancement of ATF4-induced transcription. In fact, there was a trend toward a greater enhancement by ATF3ΔZip3 with increasing ATF4 expression levels. These results demonstrate that the putative ATF3ΔZip3 protein alone has a minimal effect on basal ASNS promoter activity, but acts in a synergistic fashion with ATF4 to increase transcription from this promoter.

ATF3-FL and the Predicted ATF3ΔZip3 Protein May Cooperatively Regulate ASNS Promoter Activity—To investigate the possible coordinated control of ASNS by ATF3ΔZip3 and ATF3-
FL, HepG2 cells were cotransfected with the ASNS-172/+51 promoter/Luc reporter plasmid along with ATF3-FL expression vectors (Fig. 9A). When the ATF3ΔZip3 cDNA was cotransfected with ATF3-FL in HepG2 cells, it resulted in an enhancement of ASNS-mediated transcription at each ATF3-FL concentration tested. These results are consistent with the observation of Hashimoto et al. (14), who observed that ATF3ΔZip2 requires ATF3-FL to cause enhancement of transcription of a reporter gene driven by an ATF3 consensus element. Consistent with the data shown in Fig. 7, ATF3ΔZip2c
cDNA cotransfected with a repressive level of ATF3-FL (500 ng) had no effect on the ATF3-FL repression of ASNS promoter activity (Fig. 9B). To further test the coordinated effects of ATF4 and the truncated ATF3 isoforms, HepG2 cells were cotransfected with the ASNS promoter/Luc reporter plasmid along with ATF4, ATF3-Zip3, and ATF3-FL expression vectors (Fig. 9C). The results show that transfection of ATF3-Zip3 overrode the ATF3-FL repression of ATF4-mediated induction and that the net effect of ATF3-Zip3 was to enhance ATF4-induced transcription at a low plasmid level of ATF3-FL (1.0 ng) and to completely block the repression of ATF4 induction observed at higher ATF3-FL plasmid levels (e.g. see Fig. 7). Collectively, the data of Figs. 7–9 establish that ATF4 and multiple ATF3 isoforms may coordinately modulate the activity of the human ASNS promoter.

Transcription Factor Binding of ATF3-FL and ATF4 to the ASNS Promoter in Vivo—The data shown here provide evidence of a role for ATF3 in ASNS promoter regulation. To confirm that ATF3-FL binds to the human ASNS promoter in vivo, ChIP assays were performed. Chromatin fragments isolated from HepG2 cells incubated in MEM or in histidine-free MEM for 5 h were immunoprecipitated with two different amounts of the anti-ATF3-FL antibody, and then the immunoprecipitate was tested for ASNS promoter sequence (nucleotides -87 to -22) by real-time quantitative PCR. The “input” DNA produced only a single band with the expected molecular size of 65 bp (data not shown). Two independent negative controls were used for each experiment. First, immunoprecipitation with two different amounts of a nonspecific antibody (rabbit anti-chicken IgG) did not produce a significant amount of PCR product (Fig. 10, Control). Second, quantitative PCR using primers specific for a sequence within the ASNS coding region did not result in a significant amount of product either. However, PCR using an immunoprecipitate generated with the anti-ATF3-FL antibody amplified the expected ASNS promoter fragment, demonstrating in vivo binding to the ASNS promoter region containing the NSRE-1 site. Furthermore, when cells
incubated for 5 h in the absence of histidine were analyzed, the amount of ATF3-associated DNA was greater, consistent with an increase in transcription factor binding in the amino acid-deprived state (Fig. 10).

DISCUSSION

The data described in this report demonstrate the following novel observations. 1) ATF3 mRNA content and protein levels were elevated following amino acid deprivation or activation of the ERSR pathway. 2) ATF3-FL bound in vivo to the proximal promoter region of the human ASNS gene containing the NSRE-1 site. 3) Overexpression of ATF3-FL at relatively high levels repressed the enhanced transcription of the ASNS gene in response to amino acid deprivation or activation of the ERSR pathway. 4) The enhanced ATF3 mRNA content in HepG2 and HeLa cells was composed of mRNA species that code for ATF3-FL as well as two predicted novel truncated isoforms (ATF3ΔZip2c and ATF3ΔZip3). The latter are the result of alternative splicing. 5) Transient expression of ATF3-FL over a range of concentrations produced a biphasic action on ASNS promoter activity, further activating the ATF4-induced ASNS transcription at low concentrations of plasmid and repressing the ATF4-dependent induction at higher levels. 6) ATF3ΔZip2c, which has an internal deletion within the transcriptional activation domain and a truncated leucine zipper domain, exhibited increased mRNA content following histidine deprivation, but transient transfection of the corresponding cDNA had no detectable effect on ASNS transcription. 7) Amino acid limitation also increased the mRNA content of ATF3ΔZip3, which codes for a complete activation domain, but lacks much of the leucine zipper domain. 8) The putative ATF3ΔZip3 protein appears to function as a synergistic activator of ATF4-induced ASNS transcription, presumably by acting to sequester corepressors, as previously proposed for other truncated ATF3 isoforms (14, 15). 9) ChIP analysis provided the first in vivo evidence for ATF3 binding to the NSRE-1 sequence within the ASNS promoter.

ATF/cAMP-responsive element-binding protein is a subfamily of the larger bZIP (basic region/leucine zipper) transcription factor family. ATF3 is considered to be a stress response gene (13, 19), but the transcriptional consequences are different depending on whether ATF3-FL binds with other bZIP family members as a homodimer, in which case it often acts to repress transcription, or as a heterodimer, in which case it can either repress or activate transcription. Interestingly, the human ATF3 gene 5′-upstream region contains a recently identified element (5′-TGATGAAAC-3’) that differs from the ASNS NSRE-1 core sequence (5′-TGATGAAAC-3’) by only one nucleotide, and it is an ATF3-binding site responsible for autoregulating the ATF3 gene (20). The presence of an NSRE-1-like element may also explain the increase in ATF3 mRNA following histidine deprivation observed in the present report. The increase in ATF3 mRNA analyzed by Northern analysis did not reveal the complexity of alternative splicing because the mRNA lengths of the spliced variants ATF3ΔZip2c and ATF3ΔZip3 are not that different from that of ATF3-FL. The use of RT-PCR permitted analysis of the two alternatively spliced products and revealed that all three of these mRNA species were increased following amino acid limitation. Although the 5′-upstream region of the human ATF3 gene does not contain an ERSR element (21), there is a sequence (5′-TGACGT-3′, nucleotides −90 to −85) that matches the consensus binding site for ATF6 (22). ATF6 is an ERSR mediator that could increase ATF3 expression during ER stress (23, 24). Our results are consistent with the proposal that the ATF3 promoter, containing both an NSRE-1-like sequence and a potential ATF6-binding site, is responsible for the nutrient-dependent regulation of ATF3 mRNA expression. Confirmation of this hypothesis will require promoter analysis of the human ATF3 gene.

In this study, ChIP assays supported the hypothesis that the NSRE-1 site within the ASNS promoter binds ATF3-FL, although it is not clear whether ATF3-FL interacts directly with the DNA or indirectly as a complex with C/EBPβ or ATF4, both of which also bind at this site (10, 11). The NSRE-1 sequence differs from a C/EBP-ATF composite site within the human CHOP (C/EBP homologous protein) gene that is known to mediate induction of the gene in response to amino acid deprivation (25). By electrophoresis mobility shift assay, Fawcett et al. (26) have demonstrated that ATF3 binds to the CHOP C/EBP-ATF site in vitro and that overexpression of ATF3 represses both the basal and ATF4-induced transcription from a promoter construct containing this element. However, the functional action of ATF3-FL on the ASNS promoter activity appears to be complex. Transient expression of ATF3-FL alone caused an inhibition of basal ASNS promoter activity in cells maintained in complete MEM, consistent with its known repressor activity when present as a homodimer (19). Overexpression of ATF3-FL caused a biphasic response when coexpressed with ATF4, yielding further activation at lower plasmid levels and then antagonistic repression of ATF4 action at higher levels of plasmid. The initial stimulation by ATF3-FL may be due to a “squeezing” effect mediated by initially removing corepressor molecules from DNA-binding sites (27). Other investigators have also reported complex regulation involving ATF3. For example, Chen et al. (15) have also shown that ATF3 can both repress and activate, depending on the expression level. It is also conceivable that, in “fed” cells, exogenous ATF3-FL protein may form a heterodimer with endogenous C/EBPβ at lower ATF3-FL expression levels to cause enhanced transcriptional activity of the ASNS promoter at a time when ATF4 is not present and C/EBPβ is not yet upregulated. At higher levels of ATF3-FL expression, because of the limited amount of endogenous C/EBPβ in fed cells, excess ATF3-FL protein may form a homodimer and therefore cause an inhibition of ASNS promoter activity. ATF3 has been shown to form heterodimers with ATF2, c-Jun, JunB, and JunD (13), and these heterodimers can act as either activators or repressors depending upon the promoter. Collectively, our present data provide more evidence indicating that the interaction of multiple transcription factors contributes to the nutrient-sensing mechanism associated with the NSRE-1-binding site within the ASNS promoter.

In higher eukaryotes, alternative splicing is a mechanism that permits the number of functionally diverse proteins expressed by an organism to exceed the number of genes contained in the genome. Three previously identified alternative ATF3 mRNAs, ATF3ΔZip (15) and ATF3ΔZip2a and ΔZip2b (14), all stimulate transcription of a reporter gene when transiently expressed. ATF3ΔZip was identified in HeLa cells stimulated by serum (15), whereas ATF3ΔZip2a and ATF3ΔZip2b were identified in primary human umbilical vein endothelial cells after treatment with several stress-associated stimuli (14). The putative ATF3ΔZip isoforms lack a functional leucine zipper domain and therefore do not appear to bind to DNA, but do retain corepressor-binding activity. Therefore, it has been proposed that they cause transcriptional activation indirectly by sequestering corepressor proteins. In support of this proposal, transient expression of these ATF3ΔZip isoforms can activate promoters that lack an ATF3-binding site (15). Under our experimental conditions, we did not detect expression of the three previously reported mRNA species (ATF3ΔZip, ATF3ΔZip2a, and ATF3ΔZip2b) in HepG2 cells. However, the expression of two novel ATF3 clones (ATF3ΔZip2c and
ATF3ΔZip3) was detected. RT-PCR was used to confirm that these two new isoforms, but not ATF3ΔZip, ATF3ΔZip2a, or ATF3ΔZip2b, are expressed in primary cultures of normal human hepatocytes and are regulated in abundance by amino acid deprivation (data not shown). Whereas transfection with ATF3ΔZip2c had no detectable regulatory effect on ASNS expression, ATF3ΔZip3 stimulated transcription from the ASNS promoter, presumably by sequestering away the corepressor from the promoter, as mentioned above. Interestingly, Hashimoto et al. (14) have shown that exogenously delivered ATF3ΔZip2 actually requires the coexpression of ATF3-FL to initiate promoter activation. Such complex interactions between the ATF3 family members may also contribute to the biphasic response of ATF3-FL to the ASNS promoter. Unfortunately, there are no commercially available antibodies to preferentially detect the predicted truncated isoforms, so differential ChIP analysis was not possible.

The data presented in this report demonstrate that ATF3 expression is enhanced following activation of either the amino acid response or ERSR pathway, and ChIP analysis documented that ATF3-FL binds to the human ASNS promoter in vivo. The NSRE-1 site within the ASNS promoter has already been shown to bind ATF4 and C/EBP, and the present data illustrate that ATF3 gene products are also involved in the control of ASNS expression. Following amino acid deprivation, multiple ATF3 mRNA species are produced at elevated levels; and based on transient expression, both ATF3-FL and at least one of the predicted truncated isoforms appear to participate in the subsequent transcriptional regulation of the ASNS gene. It will be of interest to determine the mechanism of amino acid limitation on the activation of the ATF3 gene as well as the possible role in regulating the differential splicing events that favor a relative increase in the synthesis of the ATF3ΔZip3 mRNA. Although additional details regarding the interactions of these proteins with other transcription factors remain to be established, these results underscore the contribution of bZIP transcription family members to nutrient regulation of gene expression in mammalian cells.

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