Activation of the Unfolded Protein Response Pathway Induces Human Asparagine Synthetase Gene Expression*

(Received for publication, June 24, 1999, and in revised form, August 26, 1999)

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The gene for the amino acid biosynthetic activity asparagine synthetase (AS) is induced by both amino acid and glucose deprivation of cells. The data reported here document that the human AS gene is induced following activation of the Unfolded Response Pathway (UPR), also known as the Endoplasmic Reticulum Stress Response (ERSR) in mammals. Increased AS transcription occurs in response to glucose deprivation, tunicamycin, or azetidine-2-carboxylate, all known to activate the UPR/ERSR pathway. Previously identified ERSR target genes contain multiple copies of a single highly conserved cis-element. In contrast, the human AS gene does not contain the ERSR element, as it has been described for other responsive genes. Instead, AS induction requires an Sp1-like sequence, a sequence previously shown to be associated with amino acid control of transcription, and possibly, a third region containing no consensus sequences for known transcription factors. Oligonucleotides covering each of these regions form DNA-protein complexes in vitro, and for some of the amount of these complexes is greater when nuclear extracts from glucose-starved cells are tested. These results document that a wider range of metabolic activities are activated by the UPR/ERSR pathway than previously recognized and that genomic elements other than those already described can serve to enhance transcription of specific target genes.

Although the cellular significance of this carbohydrate-dependent control of the AS gene is not fully understood, it is known that for cells lacking sufficient AS activity asparagine deprivation results in cell cycle arrest (6, 7) and induction of apoptosis (8, 9). Maintenance of asparagine levels via induction of AS activity may play a key role in the response to the cellular stress of carbohydrate limitation. A number of genes are increased in their transcriptional rate following glucose deprivation including, GRP78, GRP94, protein disulfide isomerase, calreticulin, and the transcription regulatory factor C/EBP homology protein (chop)/growth arrest and DNA damage protein 153 (gadd153) (10). Collectively, the changes following glucose starvation are the result of a cellular recognition of protein accumulation within the endoplasmic reticulum (ER), called the Unfolded Protein Response (UPR) in yeast (11, 12) or the ER Stress Response (ERSR) in mammalian cells (13, 14). The transcription of this same set of genes can be increased by treatment of cells with the glycoprotein biosynthesis inhibitor tunicamycin or with amino acid analogs that incorporate and cause improper folding, such as the proline analog azetidine-2-carboxylate (Aze) (15). The consensus cis-element responsible for the UPR in yeast (5'-CAGCGTG-3') (16) and the corresponding ERSR element in mammalian cells (5'-CCAT-N9-CCACG-3') (13, 14) are significantly different, although many, if not most, of the corresponding genes are targets for both.

Given the observed increased transcription of the human AS gene following glucose deprivation (5), the present experiments were designed to test the hypothesis that AS is a target gene of the UPR/ERSR. If AS is a UPR/ERSR target, transcription should be enhanced by recognized activators of the pathway, such as tunicamycin or Aze (15). The Northern analysis reported here indicates that either tunicamycin or Aze are as effective as glucose starvation in increasing AS mRNA content. Furthermore, tunicamycin was able to replace glucose deprivation as a way to activate the transcription of a reporter gene under the control of the AS 5'-flanking sequence. Thus, these data document that the AS gene is responsive to several known activators of the UPR/ERSR pathway. Electromobility shift analyses and promoter deletions within the proximal 200 bp of the human AS promoter indicate that there are distinct regions of sequence required for a maximal UPR/ERSR response by this gene.

EXPERIMENTAL PROCEDURES

Cell Culture—Human HepG2 hepatoma cells were obtained from the American Type Culture Collection (ATCC) and maintained in minimal essential medium (MEM) as described (5). To test for induction of AS mRNA content, cells were incubated in glucose-free MEM, complete MEM medium, MEM containing 5 μg/ml tunicamycin, or MEM containing 5 mM Aze for 12–18 h, during which the media contained 10% dialyzed fetal bovine serum.

RNA Isolation and Northern Analysis—Total cellular RNA was...
isolated and subjected to Northern analysis on a 1% agarose gel (5). The cDNA probes for human AS, GRP78, and ribosomal protein L7a, described previously (5), were radiolabeled with [α-32P]dCTP using a random primers labeling kit according to the instructions of the manufacturer (Life Technologies, Inc., Gaithersburg, MD) and purified by G-50 column (Amersham Pharmacia Biotech). DNA fragments were quantified by densitometry after demonstrating that the absorbance units were within the linear range of the film. All experiments were repeated with independent RNA preparations to show reproducibility.

Transient Expression and Mutagenesis—HepG2 hepatoma cells were transfected with a reporter construct (p0GH), obtained from Nichols Institute Diagnostics, Inc. (San Juan Capistrano, CA) that contains the complete gene sequence for human growth hormone (GH), including a potential transcription initiation site (inr) sequence (17, 18) at the known transcription start site, but lacking a promoter (19). To investigate transcriptional control by AS-specific sequences, the constitutive (UPR unresponsive) mouse metallothionein promoter (MTT) (20) or portions of the human AS promoter (as indicated in each figure) were subcloned in front of the GH sequence using the BamHI site within the p0GH vector. With the exception of the promoter and the first nucleotide of the transcript, the entire GH genomic sequence is contained within the p0GH vector. The AS promoter sequences and the site-directed mutations were generated by PCR using primers based on sequence from a human genomic clone obtained and characterized by our laboratory. Our sequence agrees with the sequence of the entire AS gene and its flanking regions obtained by sequencing of a PAC clone (clone DJ1090P18, GenBank™ accession number AC00536) and submitted to GenBank by R. Waterston as part of the Human Genome Project.

A batch transfection technique was employed using HepG2 cells grown to about 75% confluence. A ratio of 10 μg of DNA per 60 μl of Superfect reagent (Qiagen, Germany) per 6.6 × 10^6 cells/200-mm dish was used in each transfection. A 10-μg aliquot of DNA was incubated with 60 μl of Superfect for 10 min at room temperature in MEM alone. The MEM was removed, and the cells were washed once with phosphate-buffered saline and then incubated with the transfection mixture for 3 h at 37 °C in 5.0 ml of MEM containing fetal bovine serum, bovine serum albumin, and antibiotics. After transfection, the cells were washed once with phosphate-buffered saline, fresh culture medium was added, and the cells were cultured for 24 h. Each 100-mm dish of HepG2 cells was then split to four or five 60-mm dishes so that, within each experiment, cells incubated in either glucose-containing, glucose-free, or tunicamycin-containing medium came from the same plate of HepG2 cells. This procedure eliminates the concern about transfection efficiency between the experimental treatments. After another 24 h of culture, the cells were incubated for 12–18 h in MEM lacking glucose or complete MEM containing 5 μg/ml tunicamycin or 5 mM Aze for 18 h, the cellular content of AS mRNA was increased significantly (Fig. 1A). The response to glucose deprivation is consistent with our previous report (5) and served, in this context, as a positive control. The Northern blots were stripped and reprobed with cDNAs specific for GRP78, which served as a positive control for the UPR/ERSR pathway, and for the ribosomal protein rpL7a, which served both as a negative control and to document equal loading between gel lanes (Fig. 1A). To determine whether or not the induction by tunicamycin treatment and glucose deprivation were additive, HepG2 cells were incubated under each condition separately or in MEM medium both lacking glucose and containing tunicamycin (Fig. 1B). The cells were exposed to both conditions simultaneously, the degree of enhancement in AS mRNA was approximately the same as when the same conditions were present alone (Fig. 1C). These results support, but do not prove, the hypothesis that the two processes converge on a common step, if not identical mechanisms. Quantitation and reproducibility of the response to these UPR/ERSR activators was accomplished by treating three independent sets of cells, measuring the AS mRNA content by hybridization of a dot blot, and quantitating the results with a phosphomager (Fig. 1C).

To establish that the observed increase in steady state AS mRNA content following tunicamycin treatment was transcriptionally mediated and to localize potential genomic cis-element regions, a transient transfection protocol was used. HepG2 cells were transfected with a reporter construct containing the entire genomic sequence for human GH (19) in conjunction with: 1) no promoter (p0GH), 2) the mouse MTT promoter (20), or 3) specific fragments of the AS promoter (Fig. 2A). Transcription was significantly enhanced by glucose deprivation when 10 kilobase pairs of the human AS 5′-flanking sequence was present.
FIG. 1. AS mRNA content is increased in response to several independent activators of the UPR/ERSR pathway. HepG2 human hepatoma cells were incubated in MEM, MEM – Glc, MEM + tunicamycin (5 μg/ml), or MEM + Aze (5 μM) (panel A) or MEM, MEM – Glc, MEM + tunicamycin, or MEM – Glc + tunicamycin (panel B) for 18 h. Total cellular RNA was isolated and subjected to Northern analysis (15 μg of RNA/lane). The resulting blots were hybridized sequentially with radiolabeled probes for AS, rpL7α, and GRP78. To assess the variation of the response, in a separate series of experiments, hybridization of AS mRNA by dot blot (20 μg/spot) was performed on triplicate samples and quantitated by phosphoimager analysis (panel C).

FIG. 2. Sequences within the human AS proximal promoter region mediate the transcriptional induction in response to activation of the mammalian ERSR pathway. HepG2 human hepatoma cells were transfected with a human GH reporter construct lacking a promoter (p0GH), containing the ERSR-unresponsive MTT promoter, or the indicated regions (−3121/+51, −615/+51, or −173/+51) of the AS 5′-flanking sequence. After incubating the cells for 12 h in MEM, MEM – Glc (panel A), or MEM + tunicamycin (panel B) to induce the ERSR pathway, expression of the GH reporter mRNA was monitored by Northern analysis (20 μg of RNA/lane). Panels A and B illustrate a representative experiment, whereas panel C represents quantitation of four independent experiments. In panel C, the construct showing the highest expression level (−3121/+51 for the −Glc series and −173/+51 for the + tunicamycin series) was set to 100% and averages ± S.D. for the other values were normalized. To confirm the ERSR pathway activation and to assess the lane loading consistency, the blots were rehybridized with radiolabeled probes to measure the endogenous AS, GRP78, and rpL7α mRNA content. To illustrate the transfection efficiency between constructs, lacZ mRNA was measured.

cis-elements, deletions were made to the −173/+51 bp sequence from both the 5′ and 3′ directions (Fig. 3). Deletion of the AS 5′ sequence from nt −173 to −111 had no effect on either basal or ERSR-mediated transcription. However, reduction of the sequence to nt −90 or less caused a decrease in transcription, consistent with the presence of an Sp1 consensus sequence located at nt −106 to −97 (25). Given that the genomic GH reporter sequence lacks only one nucleotide from the native transcript sequence (19) and retains an Inr-like sequence that appears to serve as a transcription start site (17, 18), 3′-deletions of the AS promoter were also possible (Fig. 3B). Sequential removal of AS promoter sequence from the 3′...
direction back to nt −34 resulted in a partial loss of basal transcription, but the ERSR-induced transcription remained evident (Fig. 3B). Collectively, these deletion studies indicate that, by transient transfection studies, the sequences required for effective expression of the AS gene following ERSR activation are contained within the −111 to −34 proximal promoter sequence.

Interestingly, the TATA sequence (5′-TATAA-3′) at nt −29 to −25 appeared to be unnecessary for basal transcription, at least in the context of the GH reporter gene. To investigate further the functionality of this sequence in the context of the AS promoter, the AS sequence 5′-TATAA-3′ was mutated to 5′-ACTCA-3′ within the −173 to +51 construct (labeled −173/+51/TATA in Fig. 3B). Neither basal nor induced transcrip-
tion was altered by mutation of the TATAA sequence, suggesting that this sequence is not functionally required for AS expression. Genes lacking a TATA box often use an Inr initiator element in conjunction with multiple Sp1 sites to localize the transcription start site (reviewed in Ref. 18). The human AS has an Inr-like sequence (5'-TCAAGCT-3') at the proposed major transcription start site (7), although the functionality of this element in vivo has yet to be established.

To test in vitro for protein binding to these AS promoter sequences, electromobility shift assays (EMSAs) were performed (Fig. 4). Nuclear extracts were prepared from cells incubated for 18 h in complete MEM (+Glc) or MEM lacking glucose (−Glc) and incubated with each of three 32P-labeled double-stranded oligonucleotide probes that spanned most of the −111 to −34 region (see Fig. 5). The −112/−91 oligonucleotide probe, designed to have 6 nt on each side of a consensus Sp1 sequence (nt −106 to −97) (25), exhibited a single primary complex that could be blocked by an excess of cold competitor (Fig. 4). Preliminary experiments suggest that this band undergoes a so-called supershift when anti-Sp1 antibody is included in the incubation (data not shown). The relative amount of complex formed was the same or slightly greater when extracts from glucose-deprived cells were compared with those from glucose-fed cells.

At nt −70 to −64, the human AS promoter contains a palindromic core sequence (5'-CATGATG-3') that has been proposed to function as an amino acid response element (3). An oligonucleotide probe that contained nt −72 to −62 did not produce a product by EMSA. However, when the oligonucleotide length was increased to cover nt −79 to −53, two distinct complexes were formed. The upper complex was increased by more than 7-fold when extracts from glucose-deprived cells were used (Fig. 4). The lower complex also increased in amount when the −Glc extracts were tested, but the change (3-fold) was less than that for the larger complex. Consistent with the block substitution by Guerrini et al. (3), who documented the amino acid response activity of this region, our data show that deletion of the seven nucleotides comprising the core sequence (5'-CATGATG-3') from the −3121/+51 AS genomic fragment completely prevented the ERSR-dependent induction when tested by transient transfection.2

The deletion analysis suggested that sequences within nt −111 to −34 were necessary for activation of the AS gene by the ERSR pathway. The AS sequence from nt −49 to −30, on the top strand, contains the sequence 5'-CGTTACAGAGC-CAGTGC-3' which yields 3'-GCAAT-N6-CACGC-5' on the bottom strand, (see Fig. 5). This sequence is similar to the consensus ERSR element (5'-CCATG-N5-CACG-3') proposed by Yoshida (13) and Roy and Lee (14). However, the location of the AS ERSRE-like sequence on the bottom strand and in the opposite orientation is unusual, relative to all previously described occurrences (13, 14). The 9-base pair spacer of AS also contains a GGC element which is observed in most of the ERSR elements identified previously (14). An oligonucleotide covering the −55 to −26 AS sequence formed two complexes (Fig. 4B). The relative amount of the lower complex was unchanged by the medium glucose content, but formation of this complex exhibited sequence specificity given that self-inhibition was clearly evident, whereas an unrelated oligonucleotide sequence had no effect (Fig. 4B). The presence of a 100-fold excess of the ERSRE sequence did not prevent formation of the complex, suggesting that the protein(s) bound to this region do not recognize this consensus sequence. Although the addition of a 100-fold excess of an oligonucleotide corresponding to the consensus ERSRE element blocked the formation of the upper complex, an oligonucleotide containing an unrelated sequence was just as effective (Fig. 4B). These data suggest that formation of this minor complex is not sequence-specific. Therefore, although the deletion data indicates that the AS region between −34 and −52 is necessary for ERSR-dependent activation of the gene, identification of the specific nucleotides responsible must be confirmed by more extensive analysis.

In summary, the data presented document transcriptional control of the human asparagine synthetase gene following activation of the UPR/ERSR pathway. This report is the first demonstration of a link between amino acid metabolism and the UPR/ERSR. The physiologic importance of asparagine biosynthesis to this signal transduction process remains open for investigation. Asparagine is the N-linked carbohydrate attach-
ment site within ER-synthesized glycoproteins, so perhaps the UPR/ERSR activation of the AS gene is to ensure that asparagine limitation is not the cause of ER protein accumulation. Alternatively, both asparagine deprivation (6–9) and activation of the UPR/ERSR pathway (26) have been linked to cell cycle arrest and initiation of apoptosis. If the original signals that arise from asparagine deprivation and the UPR/ERSR represent two completely independent events, induction of AS expression may be a cellular attempt to provide sufficient asparagine so as to eliminate limiting amounts of this amino acid as the cause of the apoptotic signal.

Beyond documenting the novel observation that AS is a UPR/ERSR target, the data also provide evidence that the transcriptional elements required for control of some genes by the mammalian ERSR pathway is more complex than previously thought. Known ERSR target genes, such as the GRP family, appear to be fully activated by multiple copies of a single cis-element termed the ERSRE (13, 14). The observation that an Sp1 sequence (−106 to −97) is required for full induction of AS by the ERSR pathway is unprecedented. Interestingly, Sp1 also is reported to play a key role in regulating genes that are carbohydrate-responsive in the opposite direction, that is, that their expression is induced by the presence of glucose (27). The second AS promoter region that is implicated by the present data contains a sequence previously reported to be necessary for the transcriptional response to amino acid starvation (3). Deletion analysis shows that the core sequence (nt −70 to −64) also is necessary for activation of the AS gene by the UPR/ERSR pathway.2 Furthermore, the EMSA results of Fig. 4 document that the region forms specific DNA-protein complexes in vitro for which the amount is greater when nuclear extracts from glucose-starved cells are tested. Additional research will be necessary to fully understand the dual role of this regulatory cis-element in the cellular response to both amino acid and glucose deprivation.

Future genomic analyses, both in vitro and in vivo, will further define the specific nucleotides required for this interesting transcriptional activation of the human AS gene by the UPR/ERSR signal, whereas knockdown or knockout approaches will address the physiological role of asparagine biosynthesis in the pathway.

Acknowledgments—We thank Dawn Beachy for secretarial support and other members of the laboratories for technical advice and helpful discussion.

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