Characterization of the nutrient-sensing response unit in the human asparagine synthetase promoter

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Transcription from the human asparagine synthetase (*A.S.*) gene is increased in response to either amino acid (amino acid response) or glucose (endoplasmic reticulum stress response) deprivation. These two independent nutrient-sensing pathways converge on the same set of genomic *cis*-elements, referred to as nutrient sensing-response elements (NSREs) 1 and 2, within the A.S. promoter. The present report uses single-nucleotide mutagenesis to confirm that both NSRE-1 and NSRE-2 are absolutely required for gene activation and to identify the boundaries of each binding site. The core sequence of the NSRE-1 site is contained within nucleotides −68 to −60 and the NSRE-2 core sequence is within nucleotides −48 to −43. Through insertion or deletion of 5–10 nucleotides in the intervening sequence between NSRE-1 and NSRE-2, transient transfection studies with an A.S. promoter/reporter gene construct showed that the 11 bp distance between these two elements is critical. These results document that the optimal configuration is with both binding sites on the same side of the DNA helix, only one helical turn away from each other and the data provide support for the hypothesis that a larger multi-protein complex exists between the binding proteins for NSRE-1 and NSRE-2. The data also illustrate that the combination of NSRE-1 and NSRE-2, referred to as the nutrient-sensing response unit (NSRU), has enhancer activity in that it functions in an orientation- and position-independent manner, and conveys nutrient-dependent transcriptional control to a heterologous promoter.

Key words: amino acid, endoplasmic reticulum stress response, gene expression, metabolite control, transcription, unfolded protein response.

INTRODUCTION

A number of cellular activities are increased following amino acid deprivation [1,2], and among these is asparagine synthetase (A.S.), which catalyses the glutamine- and ATP-dependent conversion of aspartic acid to asparagine. Decades ago, Arfin and colleagues [3,4] showed that amino acid starvation of Chinese hamster ovary cells decreased the level of asparaginyl-tRNA^{Asn} with a concurrent increase in A.S. enzymic activity. Since then, it has been documented that deprivation of mammalian cells for any individual essential amino acid causes increased transcription of the *A.S.* gene [5,6], illustrating that the associated sensing mechanism, the amino acid-response (AAR) pathway, broadly detects amino acid limitation [5–7].

Guerrini et al. [8] identified a region from nt −70 to −62 within the human A.S. promoter that functioned as an amino acid-response element. More recently, Barbosa-Tessmann et al. [9–11] have demonstrated that transcription from the human *A.S.* gene is also induced by glucose deprivation, and that this activation is mediated by the endoplasmic reticulum stress response (ERSR), also known as the unfolded protein response ('UPR') pathway in yeast [12]. *In vivo* footprinting, limited mutagenesis and electrophoretic mobility-shift assay (EMSA) results showed that the region first identified by Guerrini et al. [8] is also responsible for the induction of the *A.S.* transcription following activation of the ERSR pathway [11]. The ERSR activation demonstrates that this A.S. promoter element serves in a broader capacity than simply as an amino acid-response element and, to reflect this broader substrate-detecting capability, this sequence is referred to as the nutrient-sensing response element-1 (NSRE-1) [11]. Further promoter analysis indicated that a second sequence, 11 nucleotides downstream from NSRE-1, is also required for activation by both the AAR and the ERSR pathways. This sequence is referred to as the NSRE-2 site, and, to underscore the collective function of these two sequences as a unit, the term nutrient-sensing response unit (NSRU) has been coined.

The data presented in this report provide characterization of the NSRU. The results of single-nucleotide mutagenesis define the boundaries of the NSRE-1 and NSRE-2 elements and other promoter constructs show that the NSRU functions as an enhancer element in that it mediates activation of the *A.S.* gene when present in either orientation and regardless of location, including when present downstream of the transcription start site in an intron. The 5′-to-3′ relationship and the distance between previously identified [13] upstream GC-rich sequences and the NSRU are not critical. On the other hand, the sequential order of the NSRE-1 and NSRE-2, that is NSRE-1 located 5′ to the NSRE-2, is required and their order cannot be reversed. The A.S. promoter region bounded by NSRE-1 and NSRE-2, including the 11 bp intervening sequence between them, is highly conserved across mammalian species and the distance between NSRE-1 and NSRE-2 cannot be altered substantially in length.

MATERIALS AND METHODS

Cell culture

Human hepatoma HepG2 cells were cultured in minimal essential medium (MEM) pH 7.4, supplemented to contain 25 mM

Abbreviations used: AAR, amino acid response; A.S., asparagine synthetase; NSRE, nutrient-sensing response element; NSRU, nutrient-sensing response unit; EMSA, electrophoretic mobility-shift assay; ERSR, endoplasmic reticulum stress response; G.H., growth hormone; MEM, minimal essential medium; ATF4, activating transcription factor 4; C/EBPβ, CCAAT/enhancer-binding protein β; TK, thymidine kinase.

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$NaHCO_3$, 4 mM glutamine, 10 μg/ml streptomycin sulphate, 100 μg/ml penicillin G, 28.4 μg/ml gentamycin, 0.023 μg/ml *N*-butyl-*p*-hydroxybenzoate, 0.2 % (w/v) BSA and 10 % (v/v) fetal bovine serum. Cells were maintained at 37 °C in a 5 % CO_2/95 % air incubator.

Transient transfection

To test the transcriptional capability of individual promoter constructs, a batch-transfection protocol was performed using a growth hormone (*G.H.*) reporter gene, as described previously [10]. Use of the p0GH vector, which contains the entire *G.H.* gene (including introns) as a reporter [14], and the construct containing the A.S. promoter fragment of nt $-173/+51$, have been described previously [9,11]. The HepG2 cells were seeded on 60 mm dishes (2.6×10^6 cells) 24 h before transfection. For each transfection, 5 μg of the wild-type or mutated $-173/+51$ A.S. promoter fragment, linked to the *G.H.* reporter gene, was used along with 5 μg of the co-transfection control plasmid, which was the pcDNA3.1 vector containing the *lacZ* gene driven by the cytomegalovirus promoter.

A batch-transfection technique was employed using HepG2 cells grown to about 75 % confluence. A ratio of 10 μg DNA/60 μl of Superfect reagent (Qiagen, Hilden, Germany) per 2.6×10^6 cells/60 mm dish was constant 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, cells washed once with PBS, and then incubated with the transfection mixture for 3 h at 37 °C in 5.0 ml of MEM containing fetal bovine serum, BSA and antibiotics. After transfection, cells were washed once with PBS, fresh culture medium was added and the cells were cultured for 24 h. Each 60 mm dish of HepG2 cells was then split into four 60 mm dishes, so that within each experiment cells incubated in complete MEM, MEM lacking glucose or MEM lacking histidine came from the same transfected cell population. After transfection (24 h) the cells were divided one to four and then after another 24 h of culture the cells were transferred to fresh complete MEM, glucose-free MEM or histidine-free MEM for 16–18 h, each supplemented with 10 % dialysed fetal bovine serum. Using this batch-transfection protocol, cells exposed to the two different medium conditions arose from the same initial transfection dish, thus eliminating transfection efficiency as a variable in the starvation response [10]. Each experiment was repeated with multiple batches of cells. When three or more independent transfections were performed, the data were evaluated statistically and are reported as means $\pm$ S.E.M. Total cellular RNA was isolated using an RNeasy Mini Kit according to the procedure described by the supplier (Qiagen, Valencia, CA, U.S.A.). ^{32}P-Radiolabelled cDNA probe synthesis for *G.H.* and *lacZ*, as well as the Northern analysis protocol, were performed as described previously [15].

Sequence alignment

The proximal promoter sequences for the mouse, rat, hamster and human *A.S.* genes were aligned using the ClustalW software available at http://www.ch.embnet.org/software/ClustalW.html and the output was generated with Boxshade Software (version 3.21) available at http://www.ch.embnet.org/software/BOX_form.html. The GenBank accession numbers used to obtain the sequences were: mouse, AF262321; rat, NW_043740; hamster, M27838; and human, AF239815.

RESULTS

Defining the NSRE-1 and NSRE-2 boundaries

During a previous investigation, dimethyl sulphate *in vivo* footprinting had documented that the human A.S. promoter region immediately upstream of the major transcription start site contains six putative protein-binding sites [11]. Of these six sites, the five that contribute to nutrient control of the human *A.S.* gene are shown in Figure 1: three GC boxes (GC-I, GC-II and GC-III) and two NSREs (NSRE-1 and -2). The three GC boxes serve to maintain the level of basal transcription and to permit maximal activation of the *A.S.* gene following amino acid or glucose limitation [13]. For the other two regulatory sites identified by *in vivo* footprinting, NSRE-1 and NSRE-2, protein binding was enhanced in response to activation of either the AAR or ERSR pathways. However, the exact boundaries and the core sequence of the NSRE-1 and NSRE-2 sites have not been established. The first 150 bp of the human A.S. promoter sequence was used to align homologous sequences from the A.S. promoter regions of the mouse, rat and hamster (Figure 1B). As shown by the capital letters in the consensus sequence, there was a high degree of identity within all four species for the GC-II and G-III sites, but less so for GC-I. The core sequences (as established by the mutagenesis described below) of both NSRE-1 and NSRE-2 are completely identical across these four species, and interestingly so is the intervening 11 bp sequence (Figure 1B). In contrast, the flanking sequence on either side of the NSRU was not as highly conserved.

To define the boundaries of the NSRU and to establish the core nucleotides for NSRE-1 and NSRE-2, single-nucleotide mutagenesis was performed across the entire region of these two sites in the context of the $-173/+51$ A.S. promoter–G.H. reporter construct. Transcription was assayed following transient transfection and subsequent incubation of the cells in MEM, MEM lacking histidine or MEM lacking glucose (Figure 2). For many of the nucleotides only a single mutant was prepared but, after the first round of mutagenesis, to better define the apparent edges of the NSRE-1 and NSRE-2 core sequence, both transition and transversion mutations were made. The set of mutations that best defined the boundaries are shown in Figure 2. The percentage inhibition caused by mutation of individual nucleotides within NSRE-1 or NSRE-2 was sometimes slightly different depending on whether basal or starvation-activated transcription was assayed. These variations may reflect different proteins bound in fed versus starved states, but given that mutagenesis of each nucleotide within the two binding sites suppressed both basal and activated transcription to some degree, inhibition under either condition was seen as indicative of a contribution and was used to define the 'core sequence'. The 'core' nucleotides for the NSRE-1 site are 5′-TGATGAAAC-3′ (nt -68 to -60), and for the NSRE-2 site the core element is 5′-GTTACA-3′ (nt -48 to -43). The A.S. NSRE-1 site has been documented to bind activating transcription factor 4 (ATF4) [16] and CCAAT/enhancer-binding protein β (C/EBPβ) [17], whereas the binding proteins for NSRE-2 have yet to be identified. In addition to amino acid limitation (i.e. histidine), the involvement of individual nucleotides within the NSRE-1 and NSRE-2 sites was confirmed by testing the effect of each of the mutants following activation of the ERSR by glucose deprivation.

The NSRU is orientation-independent

To characterize the function of the NSRU in more detail, one or two copies (nt -75 to -35), in either the forward or reverse

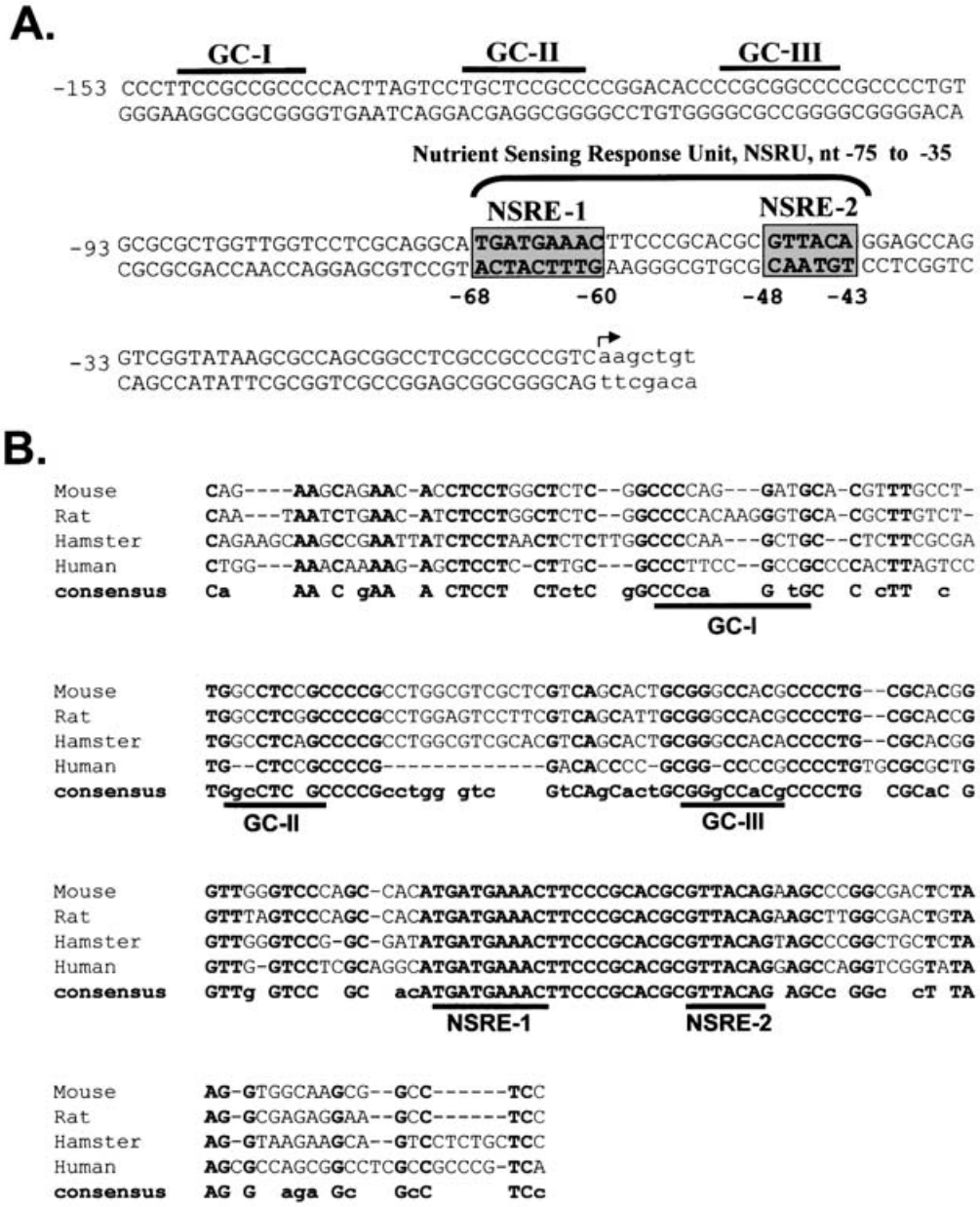


Figure 1 The proximal promoter sequence of the human *A.S.* gene

(**A**) The five A.S. promoter protein-binding sites shown contribute to nutrient control of transcription [11]. As measured by either *in vivo* footprinting or by *in vitro* EMSA, protein binding at all three GC boxes was unchanged in control and nutrient-deprived cells, whereas protein binding at NSRE-1 and NSRE-2 was enhanced by activation of either the AAR or the ERSR pathways. The shaded boxes show the boundaries of NSRE-1 and NSRE-2 based on the single-nucleotide mutagenesis shown in Figure 2. The NSRU-containing sequence, from nt −75 to −35, was used as a cassette in a number of experiments described in the remainder of this report. (**B**) The alignment of 5′ upstream regions of the mouse, rat, hamster and human *A.S.* genes is shown. The GenBank accession numbers are given in the Materials and methods section. The sequences were aligned and the output formatted as described in the Materials and methods section. Those sites for which the aligned nucleotide was identical in all four species are shown in the consensus sequence as capital letters, whereas if three of the four were identical the nucleotide is shown as a lower-case letter. The five *cis*-elements known to contribute to nutrient-regulated transcription are underlined and labelled.

direction, were inserted in front of the minimal thymidine kinase (TK)-promoter-driven *G.H.* reporter gene (Figure 3). The TK promoter alone exhibited no amino acid-dependent transcription and, conversely, the NSRU sequence in the absence of the TK promoter exhibited no significant degree of regulated transcriptional activity. However, coupling the TK promoter with one copy of NSRU, either in the forward or reverse orientation, resulted in regulated transcription such that the G.H. mRNA content was increased about 14 or 15 times the control value following histidine deprivation (Figure 3). The presence of two copies of the NSRU resulted in slightly greater transcriptional activity (24 times the control) when present in the forward direction, but was essentially the same as a single copy in the reverse orientation.

The NSRU is position-independent

To investigate the spatial relationship between the GC-rich sequences (GC-III in particular) and the NSRU, the original NSRE-1 and NSRE-2 sequences were rendered non-functional

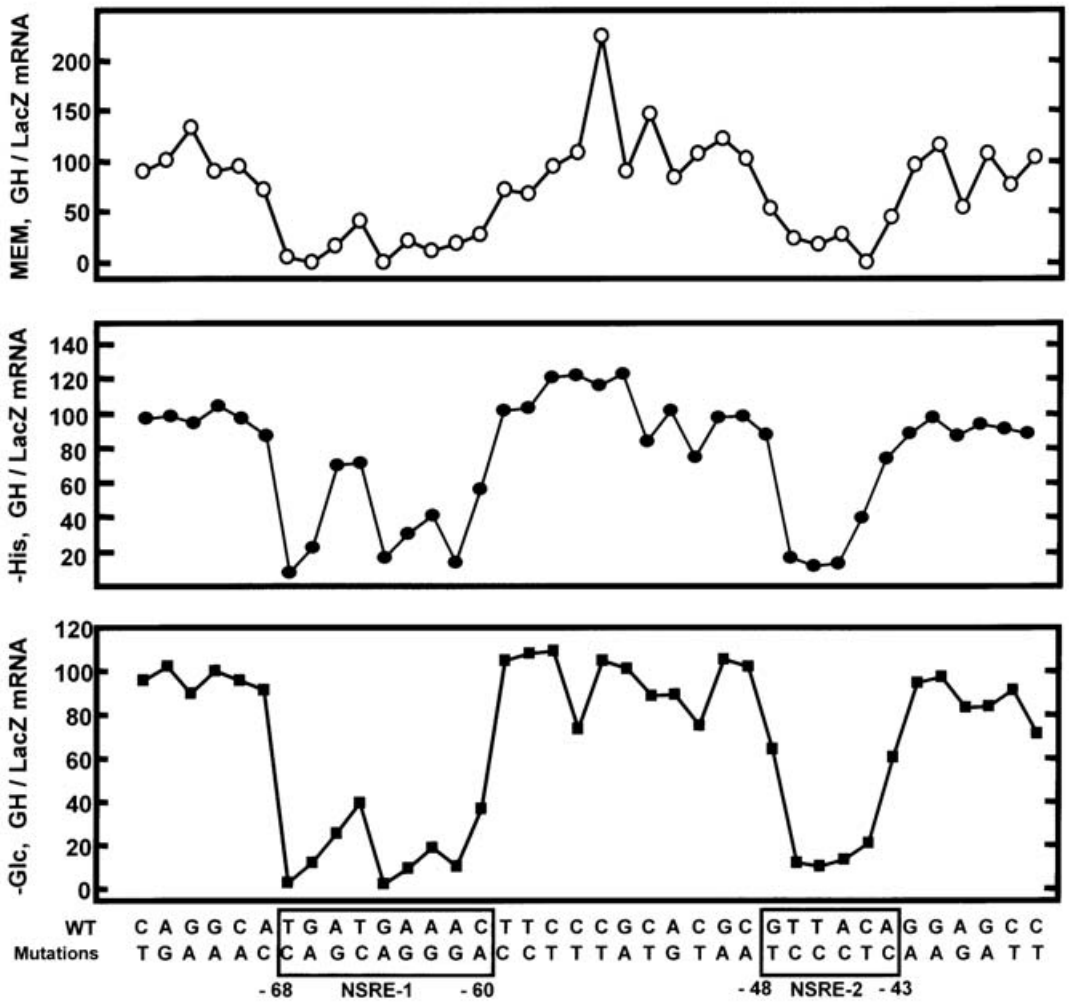


Figure 2 Boundaries of the NSRE-1 and NSRE-2 regulatory sites defined by single-nucleotide mutagenesis

Mutagenesis and transient expression of the A.S. −173/+ 51 promoter linked to the *G.H.* reporter gene was performed as described by Barbosa-Tessmann et al. [11]. Transfected HepG2 cells were incubated for 18 h in complete MEM (top panel), MEM lacking histidine (middle panel) or MEM lacking glucose (bottom panel), prior to isolation of RNA and subsequent G.H. mRNA analysis by Northern blotting. The first line of nucleotide sequence represents the wild-type A.S. promoter sequence (WT), whereas the second line shows the mutations tested. The base pairs near the edges of the NSRE-1 and NSRE-2 sites were changed to more than one nucleotide to better define these boundaries, but the graphs depict the results from the mutations indicated in the second line. The data are presented as percentages of control values obtained with the wild-type sequence.

by mutagenesis and one or two copies of the NSRU were placed between the GC-II and GC-III within the context of the A.S. genomic fragment containing nt −173/+ 51 (Figure 4). Previous studies showed that promoter deletion constructs lacking the GC-I and GC-II sites, but retaining the GC-III site, yielded transcription rates nearly equal to those constructs containing all three GC-rich elements [13]. In the native *A.S.* gene there are about 30–40 bp between the GC-III site and the NSRU (Figure 1). The present experiments were designed to determine whether the NSRU is still functional when placed 5′ to the GC-III site, testing whether or not the sequential order of GC-III and NSRU is critical and if the distance between the GC-rich sequences and the NSRU is important. Mutation of the original NSRU significantly reduced basal transcription and completely prevented the amino acid-regulated transcription (Figure 4). The basal transcription rate was different depending on the number of NSRU copies inserted. One copy of NSRU resulted in a substantially lower basal rate relative to the wild-type A.S. promoter, whereas two copies returned the basal rate to a level nearly equal to that of the control. With regard to the fold-induction of transcription following histidine deprivation, either one or two NSRU copies were equally effective (Figure 4).

The G.H. reporter plasmid (p0GH) contains the entire *G.H.* gene [14]. To test whether or not the NSRU could function at a position downstream from its original site and, in fact, at a location 3′ to the transcription start site, the NSRU was inserted at nt + 108, a location within the first intron of the *G.H.* reporter gene. Although the basal rates remained lower than the wild-type genomic fragment when either one or two copies of the NSRU was placed downstream, the induction following histidine limitation was even greater than the control (Figure 4). The presence of two copies of the NSRU within the *G.H.* intron was somewhat more effective than a single copy in mediating the induced transcription following histidine limitation.

Spacing between NSRE-1 and NSRE-2 is critical

There are 11 bp between NSRE-1 and NSRE-2, indicating that these two sites are aligned on the same side of the DNA helix.

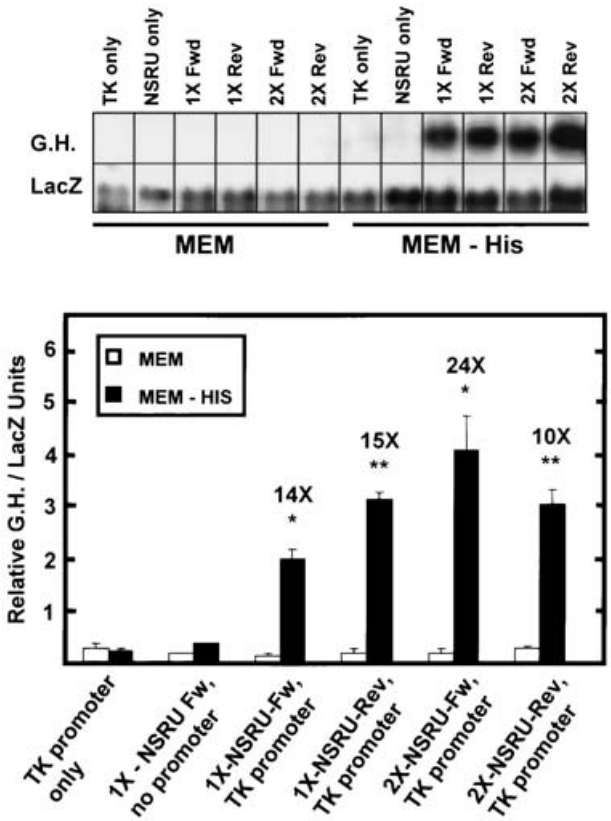


Figure 3 The NSRU is orientation-independent

HepG2 cells were transfected with the constructs indicated and in the manner described in the Materials and methods section. After 48 h, the cells were then incubated for 18 h in either complete MEM or MEM lacking histidine (–His) prior to RNA isolation and analysis for G.H. or LacZ mRNA by Northern blotting (upper panel). As indicated, the NSRU sequence (nt -75 to -35) was placed into the G.H. reporter plasmid lacking a promoter (p0GH) or placed immediately upstream of the minimal TK promoter (pTKGH) linked to the *G.H.* reporter gene [14]. The NSRU sequence was present in either one or two copies and either in the forward (Fw) or reverse (Rev) orientation. The quantification of the G.H. mRNA was normalized to the LacZ mRNA content (lower panel) as a co-transfection control and gel loading was evaluated by ribosomal RNA. Most bars represent means from three independent experiments and S.E.M. values are shown, whereas those bars without S.E.M. are means from two experiments. The starvation-dependent induction relative to the MEM value is shown above each of the NSRU constructs and the asterisks indicate statistical significance ($^{*}P < 0.05$, $^{**}P < 0.005$).

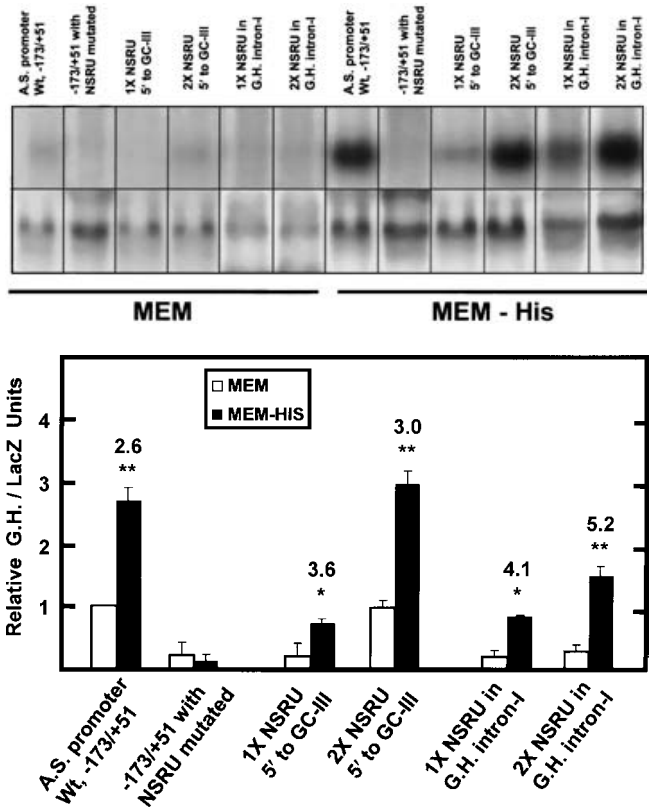


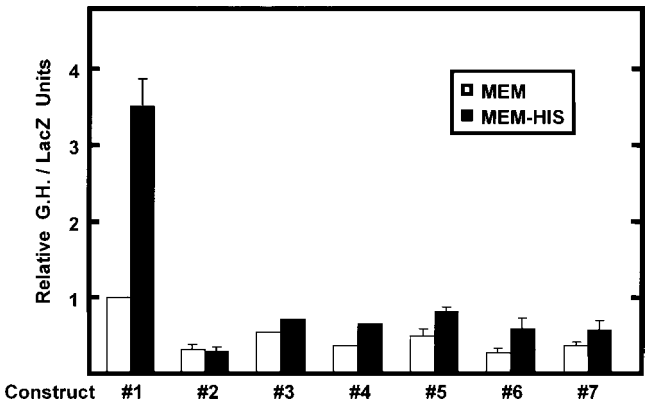
Figure 4 Function of the NSRU is position-independent

HepG2 cells were transfected with the indicated construct, cultured for 48 h, and then incubated for 18 h in complete MEM or MEM lacking histidine (–His), as described in the Materials and methods section. The transcriptional rate for each construct was measured by Northern analysis for the G.H. mRNA content (upper panel). The G.H. reporter expression was driven by the wild-type (Wt) A.S. $-173/+51$ sequence as a positive control or that same sequence with mutations (those shown in Figure 2 were used) in both the NSRE-1 (nt -64) and NSRE-2 (nt -44) to suppress the induction following amino acid limitation. This A.S. $-173/+51$ promoter construct with the original NSRU doubly mutated was then used to place one or two functional copies of the NSRU sequence (nt $-75/-35$) at nt -112 of the A.S. promoter region (between GC-II and GC-III, see Figure 1A) or at nt $+108$ of the *G.H.* reporter gene (nt $+108$ is in the first intron). The quantification of the G.H. mRNA was normalized to the LacZ mRNA content as a co-transfection control and gel loading was evaluated by rRNA (lower panel). Bars represent means from three independent experiments and the S.E.M. is shown. The starvation-dependent induction relative to the MEM value is shown above each of the NSRU constructs and the asterisks indicate statistical significance ($^{*}P < 0.05$, $^{**}P < 0.005$).

This arrangement may prove important to permit a protein complex to be formed between the factors bound at each of the sites. To directly test the importance of the spatial relationship between NSRE-1 and NSRE-2, the distance between the two elements was either increased or decreased (Figure 5). Relative to the wild-type sequence, deletion of the 5 bp from either the 5′ (Figure 5, construct no. 2) or the 3′ (Figure 5, construct no. 3) end of the 11 bp intervening region resulted in a strong inhibition of starvation-dependent activation of transcription. Doing the converse, insertion of 5 nt to extend the intervening region to 16 bp, by duplication of either the 5′ or 3′ ends of the intervening region, also resulted in suppression of the transcriptional activation by histidine limitation (Figure 5, constructs 4 and 5). To determine if placement of the two binding sites on the same side of the helix, but separated by two turns of DNA instead of one, was detrimental, insertion of 10 bp within the intervening region was tested (Figure 5, construct no. 6). Once again, a nearly complete loss of nutrient-regulated transcription was observed. Collectively, the results indicate that the distance between NSRE-1 and NSRE-2, one turn of DNA, is absolutely critical for the NSRU to function effectively. Interestingly, when the distance between NSRE-1 and NSRE-2 was kept constant (11 bp), but the order of the two elements was reversed, that is NSRE-2 was placed 5′ to NSRE-1, starvation-induced transcription was also prevented (Figure 5, construct no. 7). These data provide further evidence that spatial orientation of the two elements is essential for regulatory function.

DISCUSSION

The data presented in this report provide further characterization of a unique nutrient-sensing genomic unit that mediates transcriptional activation of the human *A.S.* gene in response to both amino acid deprivation and ER stress. This genomic unit, referred to as the NSRU, comprises two distinct but interdependent *cis*-elements, NSRE-1 and NSRE-2, and a highly conserved intervening sequence. The results described establish



Construct Number

1 **Wild Type:**
CAGGCATGATGAAACTTCCCGCACGCGTTACAGGAGCC
NSRE-1 NSRE-2

2 **Delete - 5′ spacer 5-nt:**
CAGGCATGATGAAACT CACGCGTTACAGGAGCC
NSRE-1 NSRE-2

3 **Delete - 3′ spacer 5-nt:**
CAGGCATGATGAAACTTCCC CGTTACAGGAGCC
NSRE-1 NSRE-2

4 **Insert - 5′ spacer 5-nt:**
CAGGCATGATGAAACTTCCC***TTCCC***GCACGCGTTACAGGAGCC
NSRE-1 NSRE-2

5 **Insert - 3 ′spacer 5-nt:**
CAGGCATGATGAAACTTCCCG***CACGC***CACGCGTTACAGGAGCC
NSRE-1 NSRE-2

6 **Insert 10-nt spacer:**
CAGGCATGATGAAACTTCCC***GCACGCTCCC***GCACGCGTTACAGGAGC
NSRE-1 NSRE-2

7 **Reverse order:**
ACGCGTTACAGGAGCGCCAGGCATGATGAAACTTCC
NSRE-2 NSRE-1

Figure 5 The spatial relationship between NSRE-1 and NSRE-2 is critical

HepG2 cells were transfected with the indicated A.S. promoter–G.H. reporter construct, cultured for 48 h, and then incubated for 18 h in complete MEM or MEM lacking histidine (–His), as described in the Materials and methods section. The 11 bp distance between NSRE-1 and NSRE-2 was decreased as indicated (lower panel) by deletion of the five nucleotides at either the 5′ (construct no. 2) or 3′ (construct no. 3) end of the 11 bp sequence. Conversely, the intervening sequence was increased in length by inserting a duplicate 5-nt sequence of either the 5′ (construct no. 4) or 3′ (construct no. 5) end or by duplicating 10 bp (construct no. 6) of the 11 bp sequence. A construct (construct no. 7) was also tested that contained an 11 bp intervening sequence, but the order of NSRE-1 and NSRE-2 was switched such that the NSRE-2 site was 5′ to the NSRE-1 site. The transcriptional rate for each A.S. promoter construct was measured by Northern analysis for the G.H. reporter (upper panel). The bars with S.E.M. represent means from of three independent experiments, whereas bars without S.E.M. show means from two experiments.

the following properties with regard to this regulatory unit. (i) Single-nucleotide mutagenesis throughout the region of the NSRU defined the boundaries and the core sequence of the NSRE-1 and NSRE-2 elements. (ii) The NSRU functions as an enhancer element in that it mediates activation of the *A.S.* gene when present in either orientation and regardless of location, including when present in an intron. (iii) The sequential order of, and therefore the distance between, the NSRU and the upstream GC-III sequence is not critical. (iv) The sequential order of NSRE-1 and NSRE-2, within the NSRU, is critical and cannot be reversed, nor can the distance between NSRE-1 and NSRE-2 be substantially altered in length. (v) The region bounded by NSRE-1 and NSRE-2, including the intervening sequence between them, is highly conserved across mammalian species.

In previous studies, a few selected nucleotides within the NSRU region had been mutated to support *in vivo* footprinting results and to test whether or not both NSRE-1 and NSRE-2 were required for induction of the *A.S.* gene following nutrient deprivation [11]. The data presented here significantly extend those initial observations by performing site-directed mutagenesis on each base pair within the A.S. promoter from nt −74 to −37, a region that encompasses both *cis*-elements that make up the NSRU, as well as approximately 6 bp on either side of the NSRU. These results demonstrate that the core sequence of NSRE-1 is represented by nt −68 to −60 (5′-TGATGAAAC-3′) and the NSRE-2 core sequence is from nt −48 to nt −43 (5′-GTTACA-3′). Mutagenesis of the individual nucleotides across the 11 bp intervening sequence did not result in blockade of either basal or nutrient-regulated transcription. This result is interesting given the absolute conservation of these 11 nucleotides in the mouse, rat, hamster and human genomes. A similar theme is observed in the tripartite promoter regulatory sequence, two distinct *cis*-elements separated by 9 bp of intervening sequence (5′-CCAATN$_9$CCACG-3′), that mediates the mammalian ERSR for genes such a GRP78 and other ERSR-induced molecular chaperones [18]. In this instance, TFII-I has been shown to bind to the 9 bp spacer region [19]. Although *in vivo* footprinting did not reveal a significant level of protein binding to the A.S. NSRU intervening sequence, given the high degree of conservation across species it will not be surprising if future studies reveal a contribution of this sequence to formation of the complete NSRU protein complex.

The NSRU functions as an enhancer element. Reversing the orientation of the complete NSRU (nt −75 to −35) resulted in full retention of the amino acid-regulated transcription, although reversing the order of the NSRE-1 and NSRE-2 sites within the NSRU completely blocked its activity. In addition to being orientation-independent, the NSRU is also location-independent, even functioning when placed in the first intron of the *G.H.* reporter gene. These experiments also revealed the interesting observation that the order and distance between the last of three GC boxes, known to be Sp1/Sp3-binding sites [13], and the NSRU is not critical. The presence of the last one of these Sp1/Sp3-binding sites, GC-III, contributes to both basal and regulated transcription [13]. Although GC-III is approx. 40 bp upstream of the NSRE-1 sequence in the native A.S. promoter, placing the NSRU 5′ to GC-III did not adversely effect the NSRU-mediated induction of transcription following histidine limitation. Collectively, these results indicate that the NSRU functions as an enhancer and suggest that comparable nutrient-sensing regulatory units in other genes may not be restricted to promoter locations.

The A.S. intervening region between the NSRE-1 and NSRE-2 binding sites is 11 bp in length, allowing for the proteins bound at these two sites to be on the same side of the helix and, therefore, possibly to form protein–protein interactions. The experiments described here illustrate that this distance is critical for NSRU function. However, just residing on the same side of the helix does not appear to be sufficient. Adding an additional 10 bp to the intervening region to retain the alignment of NSRE-1 and NSRE-2 on the same side of the helix, but now separated by one additional turn of DNA, resulted in a complete loss of nutrient-regulated transcription. This observation, along with the absolute requirement for both NSRE-1 and NSRE-2, leads to the speculation that the binding proteins at these two sites are bridged by a larger protein complex.

Using extracts from HepG2 hepatoma cells, proteins capable of binding to the NSRE-1 site have been identified as ATF4 [16] and C/EBPβ [17], consistent with the designation of the highly related sequence in the human *chop* gene as an ATF–C/EBP composite

site [20] which serves as an amino acid-response element [20]. Site-directed mutagenesis has identified the core sequence of the amino acid-response element within the human Chop promoter (5′-TGATGCAAT-3′) and it differs from A.S. NSRE-1 by two nucleotides [21]. The binding proteins that correspond to the A.S. NSRE-2 site have yet to be identified, but once this is achieved experiments will be possible to test the hypothesis that the 11 bp intervening sequence distance is a critical factor that may permit a larger protein complex to form between the NSRE-1 and NSRE-2 sites and possibly allow binding of bridging proteins. The high degree of species conservation of the 11 bp NSRU intervening sequence would certainly be consistent with the proposal that it is a site for specific protein binding rather than simply as a spacer to provide a given distance between NSRE-1 and NSRE-2.

In summary, the NSRU is a unique transcriptional control mechanism that permits mammalian cells to respond to multiple nutrient-sensing signal-transduction pathways. Further analysis of the molecular basis for metabolite-induced gene expression via the NSRU, and other genomic regulatory units like it, will provide valuable insight into the mechanisms used by mammalian cells to respond to their nutritional environment.

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REFERENCES

1 Fafournoux, P., Bruhat, A. and Jousse, C. (2000) Amino acid regulation of gene expression. Biochem. J. **351**, 1–12
2 Kilberg, M. S. and Barbosa-Tessmann, I. P. (2002) Genomic sequences necessary for transcriptional activation by amino acid deprivation of mammalian cells. J. Nutr. **132**, 1801–1804
3 Arfin, S. M., Simpson, D. R., Chiang, C. S., Andrulis, I. L. and Hatfield, G. W. (1977) A role for asparaginyl-tRNA in the regulation of asparagine synthetase in a mammalian cell line. Proc. Natl. Acad. Sci. U. S.A. **74**, 2367–2369
4 Andrulis, I. L., Hatfield, G. W. and Arfin, S. M. (1979) Asparaginyl-tRNA aminoacylation levels and asparagine synthetase expression in cultured Chinese hamster ovary cells. J. Biol. Chem. **254**, 10629–10633
5 Gong, S. S., Guerrini, L. and Basilico, C. (1991) Regulation of asparagine synthetase gene expression by amino acid starvation. Mol. Cell. Biol. **11**, 6059–6066
6 Hutson, R. G. and Kilberg, M. S. (1994) Cloning of rat asparagine synthetase and specificity of the amino acid-dependent control of its mRNA content. Biochem. J. **303**, 745–750
7 Jousse, C., Bruhat, A., Ferrara, M. and Fafournoux, P. (2000) Evidence for multiple signaling pathways in the regulation of gene expression by amino acids in human cell lines. J. Nutr. **130**, 1555–1560
8 Guerrini, L., Gong, S. S., Mangasarian, K. and Basilico, C. (1993) *Cis*- and *trans*-acting elements involved in amino acid regulation of asparagine synthetase gene expression. Mol. Cell. Biol. **13**, 3202–3212
9 Barbosa-Tessmann, I. P., Pineda, V. L., Nick, H. S., Schuster, S. M. and Kilberg, M. S. (1999) Transcriptional regulation of the human asparagine synthetase gene by carbohydrate availability. Biochem. J. **339**, 151–158
10 Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Schuster, S. M., Nick, H. S. and Kilberg, M. S. (1999) Activation of the unfolded protein response pathway induces human asparagine synthetase gene expression. J. Biol. Chem. **274**, 31139–31144
11 Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Siu, F., Schuster, S. M., Nick, H. S. and Kilberg, M. S. (2000) Activation of the human asparagine synthetase gene by the amino acid response and the endoplasmic reticulum stress response pathways occurs by common genomic elements. J. Biol. Chem. **275**, 26976–26985
12 Patil, C. and Walter, P. (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr. Opin. Cell Biol. **13**, 349–355
13 Leung-Pineda, V. and Kilberg, M. S. (2002) Role of Sp1 and Sp3 in the nutrient-regulated expression of the human asparagine synthetase gene. J. Biol. Chem. **277**, 16585–16591
14 Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. and Moore, D. D. (1986) Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol. Cell. Biol. **6**, 3173–3179
15 Aslanian, A. M., Fletcher, B. S. and Kilberg, M. S. (2001) Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem. J. **357**, 321–328
16 Siu, F., Bain, P. J., LeBlanc-Chaffin, R., Chen, H. and Kilberg, M. S. (2002) ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. J. Biol. Chem. **277**, 24120–24127
17 Siu, F. Y., Chen, C., Zhong, C. and Kilberg, M. S. (2001) CCAAT/enhancer-binding protein beta (C/EBPb) is a mediator of the nutrient sensing response pathway that activates the human asparagine synthetase gene. J. Biol. Chem. **276**, 48100–48107
18 Roy, B. and Lee, A. S. (1999) The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. Nucleic Acids Res. **27**, 1437–1443
19 Parker, R., Phan, T., Baumeister, P., Roy, B., Cheriyath, V., Roy, A. L. and Lee, A. S. (2001) Identification of TFII-I as the endoplasmic reticulum stress response element binding factor ERSF: its autoregulation by stress and interaction with ATF6. Mol. Cell. Biol. **21**, 3220–3233
20 Fawcett, T. W., Martindale, J. L., Guyton, K. Z., Hai, T. and Holbrook, N. J. (1999) Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate *Gadd153* expression during the stress response. Biochem. J. **339**, 135–141
21 Bruhat, A., Averous, J., Carraro, V., Zhong, C., Reimold, A. M., Kilberg, M. S. and Fafournoux, P. (2002) Differences in the molecular mechanisms involved in the transcriptional activation of the CHOP and asparagine synthetase genes in response to amino acid deprivation or activation of the unfolded protein response. J. Biol. Chem. **277**, 48107–48114

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