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

Alain Bruhat, Julien Averous, Valerie Carraro, Can Zhong, Andreas M. Reimold, Mickael S. Kilberg, Pierre Fafournoux

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 50, Issue of December 13, pp. 48107–48114, 2002

Printed in U.S.A.

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*

Received for publication, June 20, 2002, and in revised form, August 22, 2002
Published, JBC Papers in Press, September 25, 2002, DOI 10.1074/jbc.M206149200

Alain Bruhat‡§, Julien Averous‡, Valérie Carraro‡, Can Zhong¶, Andreas M. Reimold‖, Michael S. Kilberg¶, and Pierre Fafournoux‡

From the ‡Unité de Nutrition et Métabolisme Protéique, Institut National de la Recherche Agronomique de Theix, 63122 Saint Genès Champanelle, France, the ¶Department of Biochemistry and Molecular Biology, Center for Mammalian Genetics, University of Florida College of Medicine, Gainesville, Florida 32610-0245, and the ‖Rheumatic Diseases Division, University of Texas Southwestern Medical Center, Dallas, Texas 75390-8884

A promoter element called the amino acid response element (AARE), which is essential for the induction of *CHOP* (a CCAAT/enhancer-binding protein-related gene) transcription by amino acid depletion, has been previously characterized. Conversely, the human asparagine synthetase (*AS*) promoter contains two *cis*-acting elements termed nutrient-sensing response elements (NSRE-1 and NSRE-2) that are required to activate the gene by either amino acid deprivation or the endoplasmic reticulum stress response. The results reported here document the comparison between *CHOP* and *AS* transcriptional control elements used by the amino acid pathway. We first establish that the *AS* NSRE-1 sequence shares nucleotide sequence and functional similarities with the *CHOP* AARE. However, we demonstrate that the *CHOP* AARE can function independently, whereas *AS* NSRE-1 is functionally weak by itself and instead requires the presence of NSRE-2. Furthermore, *AS* NSRE-2 can confer endoplasmic reticulum stress responsiveness to the *CHOP* AARE. Using activating transcription factor-2-deficient mouse embryonic fibroblasts, we also show that lack of this transcription factor does not abolish the amino acid inducibility of *AS* transcription, but this transcription factor is necessary to obtain the full *AS* response to amino acid starvation. Collectively, these results document that there are significant differences in the molecular mechanisms involved in the transcriptional activation of *CHOP* and *AS* by amino acid limitation.

The molecular mechanisms involved in the control of gene expression in response to amino acid deprivation have been extensively studied in yeast (1, 2). In addition to specific control of genes involved in the synthesis of individual amino acids, yeast employs a general control process whereby a subset of genes are coordinately regulated by starvation of the cell for any single amino acid. In mammalian cells, specific examples of enzymes, transporters, and mRNAs that are regulated by amino acid availability have been reported (3, 4). At the molecular level, most of the results have been obtained studying the transcriptional regulation of asparagine synthetase (*AS*)[1] and *CHOP* (C/EBP homologous protein) gene expression in response to amino acid deprivation.

AS is expressed in most mammalian cells and is responsible for the biosynthesis of asparagine from aspartate and glutamine. The level of *AS* mRNA increases in response not only to asparagine starvation, but also to deprivation of any individual essential amino acid (5–7). Guerrini *et al.* (8) used promoter deletion and mutation to analyze the proximal promoter of the human *AS* gene. They identified the palindromic sequence 5′-CATGATG-3′ at nucleotides −70 to −64 as necessary for the *AS* promoter regulation by amino acid availability. Recently, Barbosa-Tessmann *et al.* (9) identified, in the *AS* 5′-flanking region, a "minimum control unit" (nt −111 to −34) that yields basal as well as endoplasmic reticulum stress response (ERSR)- and amino acid-regulated transcription. Using mutation analysis, gel shift assays, and *in vivo* footprinting experiments, Barbosa-Tessmann *et al.* demonstrated that two *cis*-elements termed nutrient-sensing response elements (NSRE-1, 5′-TGATGAAAC-3′, nt −68 to −60; and NSRE-2, 5′-GTTACA-3′, nt −48 to −43) in the *AS* promoter sequence are essential for transcriptional activation by amino acid limitation or the ERSR. NSRE-1/NSRE-2 in either direction can transfer amino acid responsiveness to a reporter driven by a minimum promoter (see Fig. 4 of Ref. 9). Moreover, gel shift experiments and overexpression of dominant-negative mutants suggest that activation of the *AS* gene by either amino acid limitation or the ERSR could involve ATF-4 and C/EBPβ binding to the NSRE-1 site (10, 11).

Dormant under normal growth conditions, the *CHOP* gene, also known as *GADD153*, is induced to high levels during the cellular stress caused by a wide variety of stresses, agents, and nutrient deprivation (12–14). The induction of *CHOP* is generally linked to activation of the ERSR, itself presumably mediated by the accumulation of malfolded proteins (15). *CHOP*

* This work was supported by a grant from the Institut National de la Recherche Agronomique (to A. B. and J. A.), National Institutes of Health Grant DK-52064 (to M. S. K.), and the Arthritis Foundation (to A. M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 33-4-7362-4150; Fax: 33-4-7362-4755; E-mail: bruhat@clermont.inra.fr.

[1] The abbreviations used are: *AS*, asparagine synthetase; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; nt, nucleotide(s); ERSR, endoplasmic reticulum stress response; NSRE, nutrient-sensing response element; ATF, activating transcription factor; ER, endoplasmic reticulum; AARE, amino acid response element; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryonic fibroblast; *TK*, thymidine kinase; ERSE, endoplasmic reticulum stress response element.

encodes a nuclear protein related to the C/EBP family of transcription factors (16). Members of the C/EBP family have been implicated in the regulation of processes relevant to energy metabolism, cellular proliferation, differentiation, and expression of cell type-specific genes (17, 18). By forming heterodimers with members of the C/EBP family, the CHOP protein can influence gene expression both as a dominant-negative regulator of C/EBP binding to one class of DNA targets and by directing CHOP·C/EBP heterodimers to other sequences (19–22).

Although many of the pathway steps linking amino acids to gene regulation remain unknown, it has been demonstrated that amino acid limitation regulates *CHOP* expression through a specific pathway independent of the ER stress signaling cascade (23). The regulation of *CHOP* expression by amino acid concentration has both transcriptional and post-transcriptional components (24). Recently, an amino acid response element (AARE) was localized between nucleotides −313 and −295 in the *CHOP* promoter. This 19-bp DNA control element, which is essential for amino acid activation of the *CHOP* promoter, can regulate a basal promoter in response to starvation of several individual amino acids (25). Through block substitution mutagenesis, the sequence 5′-ATTGCATCA-3′ was identified as the minimum core sequence essential for the *CHOP* AARE activity. The *CHOP* AARE is related to C/EBP- and ATF/cAMP response element-binding protein-binding sites and was described to bind *in vitro* to ATF-2 under starved and non-starved conditions. Using ATF-2-deficient mouse embryonic fibroblasts and an ATF-2 dominant-negative mutant, the expression of ATF-2 was shown to be essential for the transcriptional activation of *CHOP* by leucine starvation (25). In parallel, it has recently been hypothesized that the transcription factor ATF-4 could also be involved in the amino acid regulation of *CHOP* expression (26).

The objective of the work presented here was to determine whether transcriptional activation of *CHOP* and *AS* in response to amino acid limitation occurs by a common mechanism. Using mutation analysis, we first established the minimum core consensus sequence in the 9-bp *CHOP* AARE that is required to confer amino acid responsiveness as 5′-(R/C)TT(R/T)CRTCA-3′. We show that a promoter containing multiple copies of *AS* NSRE-1 is regulated in response to leucine starvation and therefore can, in this circumstance, function alone as an AARE. However, although the *CHOP* AARE and *AS* NSRE-1 share structural and functional similarities, we demonstrate that lack of ATF-2 reduces (but does not abolish) the transcriptional activation of *AS* by leucine starvation, whereas the activation of *CHOP* is completely prevented. Furthermore, if the *AS* NSRE-2 sequence is placed 10 nt downstream of the AARE core in the *CHOP* promoter, regulation by both amino acid limitation and the ERSR pathway is observed.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions—HeLa cells were cultured at 37 °C in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) (Sigma) containing 10% fetal bovine serum. When indicated, DMEM/F-12 lacking leucine was used. In all experiments involving amino acid starvation, 10% dialyzed calf serum was used. Mouse embryonic fibroblasts (MEFs) deficient in ATF-2 were produced from decapitated, eviscerated, day 14.5 ATF-$2^{0/0}$ embryos (27) using a 3T3 protocol until cells passed through crisis, typically by passage 18 (28).

DNA Transfection and Luciferase Assay—Cells were plated in 12-well dishes and transfected by the calcium phosphate coprecipitation method as described previously (24). Two micrograms of luciferase plasmid were transfected into the cells along with 0.1 μg of pCMV-βGal, a plasmid carrying the bacterial β-galactosidase gene fused to the human cytomegalovirus immediate-early enhancer/promoter region, as an internal control. Cells were then exposed to the precipitate for 16 h, washed twice with phosphate-buffered saline, and incubated with DMEM/F-12 containing 10% calf serum. Twenty-four hours after transfection, cells were amino acid-starved for 16 h. After starvation, cells were harvested in 150 μl of lysis buffer (Promega) and centrifuged at 13,000 × *g* for 2 min. Twenty microliters of the supernatant were assayed for luciferase activity (Prodemat, Anduze, France). β-Galactosidase activity was measured as described previously (29). Relative luciferase activity is given as the ratio of relative luciferase units to relative β-galactosidase units. All values are the means calculated from the results of at least three independent experiments.

Nuclear Extract Preparation—Nuclear extracts were prepared from HeLa cells as described previously (25).

Oligonucleotides—Oligonucleotides were from MWG Biotech (Ebersberg, Germany). When double-stranded oligonucleotides were required, equal numbers of moles of complementary strands were heated to 90 °C for 1 min and annealed by slow cooling to room temperature.

Gel Mobility Shift Assays—Gel mobility shift assays were performed as described previously (25). To test the effect of anti-ATF-2 antibody, 1 μl of anti-ATF-2 serum (sc-6233X, Santa Cruz Biotechnology) was added to the incubation mixture at room temperature 1 h prior to addition of the labeled probe. Each mobility shift experiment was repeated three times to confirm the reproducibility of the results.

Plasmid Constructions—TATA*TK*-LUC, containing the minimum herpes simplex virus promoter for thymidine kinase (*TK*; nt −40 to +50), was generated as previously described (25). Plasmids 1×-, 2×-, and 5×*CHOP*AARE-TATA*TK*-LUC were constructed by inserting *Sst*I-*Xho*I double-stranded oligonucleotides containing one, two, and five iterations of the *CHOP* AARE sequence, respectively, into the TATA*TK*-LUC plasmid (see Fig. 3). In the same way, one, two, and five copies of the *AS* NSRE-1 sequence (bottom strand, nt −57 to −75) (10) were inserted into the TATA*TK*-LUC plasmid, producing 1×-, 2×-, and 5×*AS*NSRE-1-TATA*TK*-LUC, respectively. Mutation series in the *CHOP* AARE sequence and deletion and mutation series in the *AS* promoter were made by inserting *Sst*I-*Xho*I double-stranded mutated sequences into the TATA*TK*-LUC plasmid. p*CHOP*-LUC (nt −649 to +91) was generated as previously described (25). Plasmid p3.4*AS*-LUC, containing a 3.4-kb fragment of the human *AS* promoter region, was generated by PCR from cloned genomic DNA (8) using *Pfu* polymerase (Stratagene) and primers and antisense primers containing appropriate restriction sites at their 5′-ends. Amplified fragments were then cloned into the pGL3-basic reporter construct (Promega) using the *Xho*I and *Hin*dIII restriction sites. All luciferase plasmid constructs were sequenced before utilization using the ABI PRISM Big Dye terminator cycle sequencing reaction kit and the ABI PRISM 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

Northern Blot Analysis—Total RNA was prepared as previously described (30). Northern blotting was performed according to the procedure of Sambrook *et al.* (31). The membranes were UV-cross-linked, and then prehybridization was carried out for 2 h at 55 °C in 50% formamide, 6× SSC, 5× Denhardt's reagent, 0.5% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 10 μg/ml yeast tRNA. The human *CHOP* cDNA (BH1) and the human *AS* cDNA were generously provided by Dr. N. J. Holbrook (32) and Dr. C. Basilico (5), respectively. *CHOP* and *AS* probes were labeled by random priming with [α-^{32}P]dCTP (Ready-To-Go DNA labeling beads, Amersham Biosciences, Uppsala, Sweden). Hybridization was carried out for 16 h at 65 °C. The membranes were washed for 15 min at 65 °C successively with 2× SSC containing 0.1% SDS, 0.5× SSC containing 0.1% SDS, and 0.1× SSC containing 0.1% SDS. Labeled bands were detected by autoradiography. Autoradiogram signals were visualized using a PhosphorImager and ImageQuant software (Amersham Biosciences). To control for either variation in the amount of RNA in different samples or loading errors, all blots were rehybridized with a DNA probe corresponding to glyceraldehyde-3-phosphate dehydrogenase mRNA. Relative *CHOP* or *AS* mRNA levels were determined as the ratio of *CHOP* or *AS* mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Analysis of Gene Expression Using Real-time Reverse Transcription-PCR—Total RNA was prepared using an RNeasy mini-kit (QIAGEN Inc.) and treated with DNase I (Amplification grade, Invitrogen) prior to cDNA synthesis. RNA integrity was electrophoretically verified by ethidium bromide staining. RNA (0.5 μg) was reverse-transcribed with 100 units of Superscript II Plus RNase H$^-$ reverse transcriptase (Invitrogen) using 100 μM random hexamer primers (Amersham Biosciences) according to the manufacturer's instructions. Primers for *CHOP* (forward primer, 5′-cctagcttggctgacagagg-3′; and reverse primer, 5′-ctgctccttctccttcatgc-3′) and *AS* (forward primer, 5′-tacaaccacaaggcgctaca-3′; and reverse primer, 5′-aagggcctgactccataggt-3′) were used and yielded PCR products 200 bp in size. To control for RNA quality and cDNA synthesis, β-actin mRNA was also amplified with forward (5′-

tacagcttcaccaccacagc-3′) and reverse (5′-aaggaaggctggaaaagagc-3′) primers.

Quantification involved the use of standard curves that had been prepared with plasmids containing specific sequences of each gene. We cloned the PCR products of *CHOP*, *AS*, and β-actin into the pGEM-T-easy vector (Promega) according to the manufacturer's instructions. For the construction of standard curves for *CHOP*, *AS*, and β-actin, pGEM-T-easy plasmids were prepared as a 10-fold serial dilution in water, from 4 ng to 0.4 pg.

PCR was carried out using a LightCycler™ system (Roche Molecular Biochemicals), which allows amplification and detection (by fluorescence) in the same tube, with a kinetic approach. For LightCycler PCRs, a master mixture of the following reaction components was prepared to the indicated final concentrations: 10.4 μl of water, 1.6 μl of $MgCl_2$ (3 mM), 1 μl of forward primer (0.5 μM), 1 μl of reverse primer (0.5 μM), and 2 μl of LightCycler-FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals). The LightCycler master mixture (16 μl) was filled in the LightCycler glass capillaries, and 4 μl of cDNA (2 ng of reverse-transcribed total RNA) were added as PCR template. Capillaries were closed, centrifuged, and placed into the LightCycler rotor. The following LightCycler experimental run protocol was used: denaturation program (95 °C for 10 min), amplification and quantification program repeated 45 times (95 °C for 15 s, 60 °C for 5 s, and 72 °C for 8 s with a single fluorescence measurement), melting curve program (69–95 °C with a heating rate of 0.1 °C/s and a continuous fluorescence measurement), and finally a cooling step to 40 °C. A negative control without cDNA template was run with every assay to assess the overall specificity. LightCycler quantification software (Version 3.5) was used to compare amplification in experimental samples during the log linear phase to the standard curve from the dilution series of control plasmids. Relative results are reported in nanograms of *CHOP* or *AS*/100 ng of β-actin. Each experiment was repeated three times to confirm the reproducibility of the results.

RESULTS

The Nucleotides Required in the CHOP AARE Core Sequence to Mediate Transcriptional Induction by Leucine Are Essential for the Binding of ATF-2—We have previously shown that the minimum core sequence in the *CHOP* AARE able to render a heterologous promoter amino acid responsive is 5′-ATTGCATCA-3′ (25). To delineate more precisely the nucleotides essential for the transcriptional activity of the *CHOP* AARE, we constructed a series of point mutants by substituting each of the 9-bp core sequence nucleotides (nucleotides N1–N9) with three different nucleotides (Fig. 1). A single copy of the 19-bp *CHOP* AARE sequence with the corresponding mutation was placed upstream of the minimum herpes simplex virus promoter for *TK*. The mutant constructs were then transiently transfected into HeLa cells, and the response to leucine was determined by luciferase activity measurements under starved and non-starved conditions. Among all the mutants, mt3 and mt4 of N1(A) in the 9-bp core sequence and substitutions of N4(G) such as mt12 and mt14 and of N6(A) such as mt19 showed responsiveness to amino acid starvation. In contrast, all substitutions of N2(T) (mt6–mt8), N3(T) (mt9–mt11), N5(C) (mt15–mt17), N7(T) (mt21–mt23), N8(C) (mt24–mt26), and N9(A) (mt27–mt29) abolished the amino acid inducibility. These results establish the minimum consensus sequence in the 9-bp *CHOP* AARE that is required to confer amino acid responsiveness as 5′-(R/C)TT(R/T)CRTCA-3′ (R = G or A).

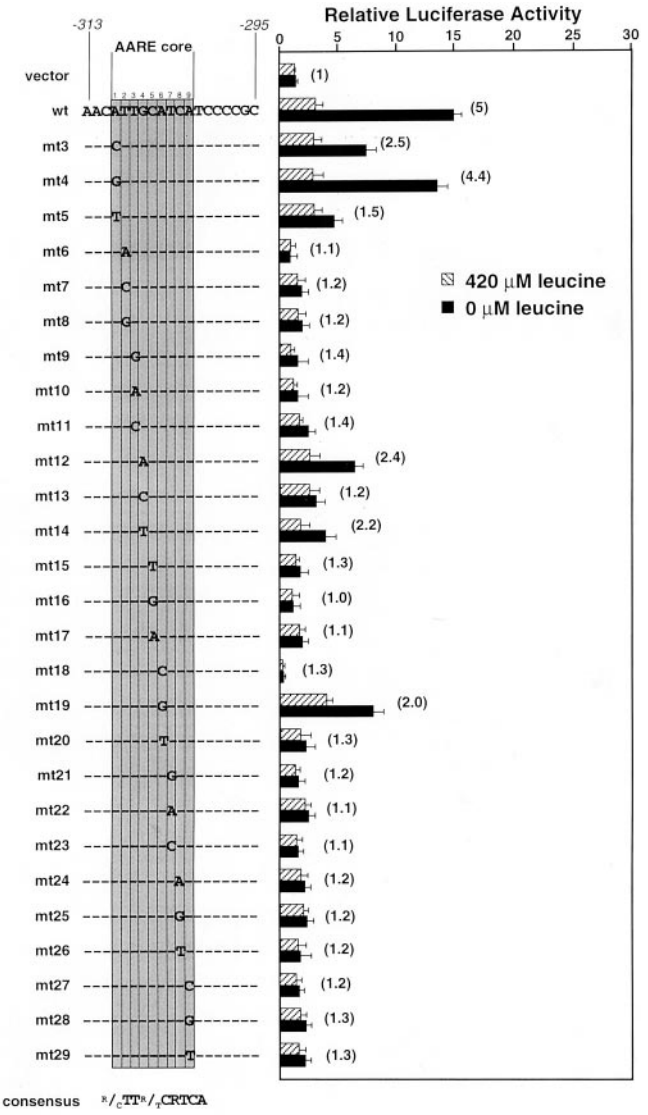

FIG. 1. **Identification of the nucleotides in the *CHOP* AARE core sequence required to mediate amino acid responsiveness.** HeLa cells were transfected with luciferase constructs containing a single copy of the native (wild-type (*wt*)) or mutant *CHOP* AARE (nt −313 to −295) inserted 5′ to the *TK* promoter. The position of the minimum AARE core sequence (nt −310 to −302) is *boxed* in *gray*, and each nucleotide of this sequence is numbered (N1–N9). Each of the nucleotides in the core sequence was changed to another nucleotide as indicated. Twenty-four hours after transfection, cells were incubated for 16 h in DMEM/F-12 with and without 420 μM leucine and then harvested for preparation of cell extracts and determination of luciferase activity. Relative luciferase activities were determined as described under "Materials and Methods." The relative -fold induction, defined as the ratio of the relative luciferase activity of leucine-starved cells to that of non-starved cells, is indicated in *parentheses* to the right of the bars. Each data point represents the mean of at least three independent experiments performed in triplicate. The resulting minimum consensus sequence is shown at the bottom (R = G or A).

The core sequence of the *CHOP* AARE has been shown to bind *in vitro* to a specific protein complex containing the transcription factor ATF-2, which plays a critical role in the transcriptional activation of *CHOP* by amino acids (25). To assess the importance of the 9-bp core sequence nucleotides in the binding of the protein complex, gel mobility shift assays were carried out with the 19-bp wild-type *CHOP* AARE oligonucleotide as a probe and mutant oligonucleotides as competitors. Among the three point mutants corresponding to one nucleotide of the AARE core sequence (Fig. 1), one mutant was chosen for electrophoretic mobility shift assay competition experiments (Fig. 2*A*). A major specific DNA·protein complex was detected after incubation of non-starved HeLa nuclear extracts with the ^{32}P-labeled *CHOP* AARE probe (*lane 1*). Oligonucleotides containing mt4 and mt12, which did not lose the amino acid inducibility (Fig. 1), abolished the binding of the protein complex to the DNA (*lanes 4* and *5* and *lanes 10* and *11*, respectively). On the other hand, oligonucleotides containing mt18, mt21, mt24, and mt27, which abolished the amino acid responsiveness (Fig. 1), did not compete for the formation of the AARE·protein complex (*lanes 14* and *15*, *16* and *17*, *18* and *19*, and *20* and *21*, respectively). Oligonucleotides containing mt6, mt9, and mt15, which lost the amino acid inducibility (Fig. 1),

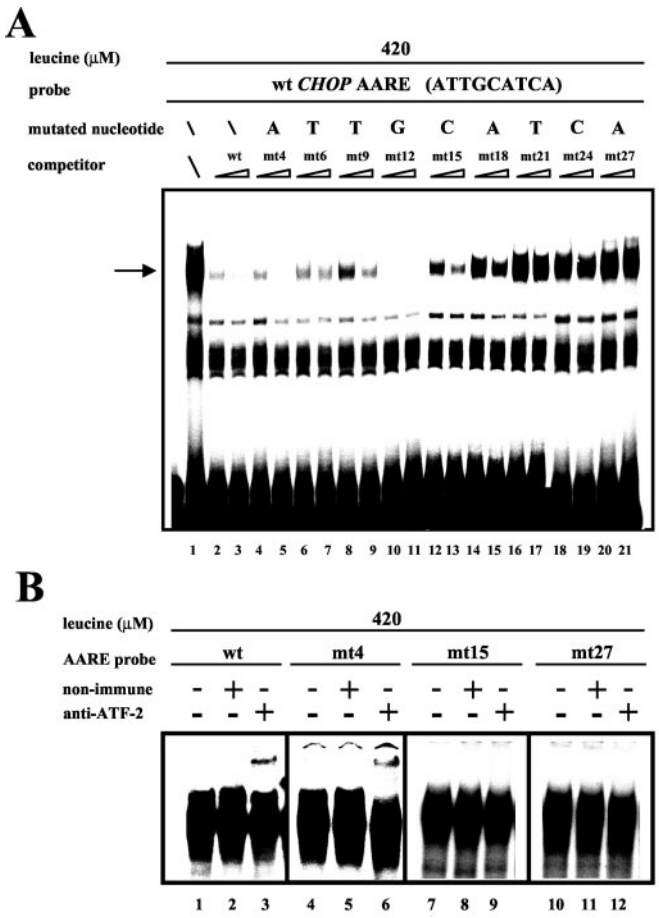

FIG. 2. **Nucleotides in the *CHOP* AARE core sequence required to confer amino acid responsiveness are essential for the binding of ATF-2.** *A*, gel mobility shift assays of nuclear extracts from HeLa cells incubated for 16 h in DMEM/F-12 containing 420 μM leucine. The 19-bp wild-type *CHOP* AARE radioactive probe carried nucleotides −313 to −295 (*wt*). The 19-bp *CHOP* AARE and different mutant AARE oligonucleotides were used as competitors at a 25- or 50-fold molar excess relative to the probe. The specific DNA·protein complex is indicated by the *arrow*. *B*, supershift assays using the antibody against ATF-2 and nuclear extracts from HeLa cells incubated for 16 h in DMEM/F-12. HeLa nuclear extracts were first incubated with 1 μl of rabbit nonimmune serum or anti-ATF-2 antiserum, and then the preincubation mixture was incubated with the 19-bp wild-type (*wt*) or mutant (mt4, mt15, and mt27) *CHOP* AARE probe as described under "Materials and Methods." The 19-bp *CHOP* AARE radiolabeled probes contained sequence −313 to −295.

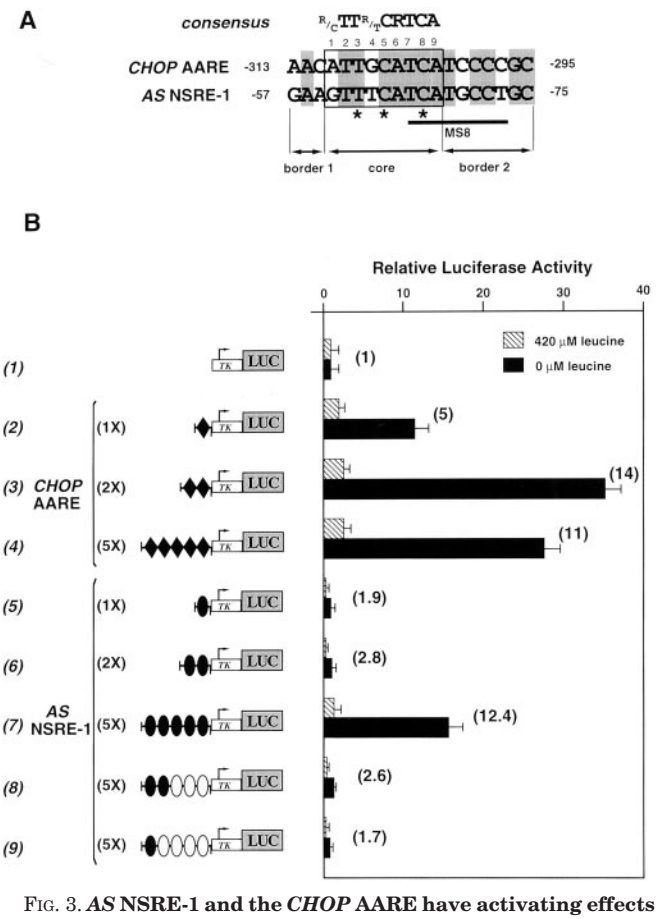

FIG. 3. ***AS* NSRE-1 and the *CHOP* AARE have activating effects on promoter activity in response to leucine starvation.** *A*, shown is a sequence comparison of the *CHOP* AARE (top strand, nt −313 to −295) with *AS* NSRE-1 (bottom strand, nt −57 to −75). Identical nucleotides are *boxed* in *gray*. The minimum AARE core sequence is *boxed*, and each nucleotide of this sequence is numbered (N1–N9). The positions of mutations in the *AS* AARE sequence that resulted in a loss of amino acid responsiveness are indicated by *asterisks* (see Fig. 8 of Ref. 9) or *underlined* (*MS8*) (see Fig. 4 of Ref. 8). The minimum core consensus sequence is represented. *B*, HeLa cells were transfected with luciferase constructs containing one, two, or five native (*black*) or mutant (*white*) copies of the *CHOP* AARE or *AS* NSRE-1 inserted 5′ to the *TK* promoter. Twenty-four hours after transfection, cells were incubated for 16 h in DMEM/F-12 with and without 420 μM leucine and then harvested for preparation of cell extracts and luciferase activity determination. Relative luciferase activities were determined as described under "Materials and Methods." The relative -fold induction, defined as the ratio of the relative luciferase activity of leucine-starved cells to that of non-starved cells, is indicated in *parentheses* to the right of the bars. Each data point represents the mean of at least three independent experiments performed in triplicate.

competed weakly for complex formation (*lanes 6* and *7*, *8* and *9*, and *12* and *13*, respectively). However, with the same amount of competitor (50-fold molar excess), oligonucleotides containing mt4 and mt12, which did not lose the amino acid inducibility, completely abolished the binding of the protein complex to the DNA. A supershift assay with the antibody against ATF-2 was performed to demonstrate directly the importance of individual nucleotides within the 9-bp core sequence of the *CHOP* AARE to ATF-2 binding (Fig. 2*B*). ATF-2 bound to the wild-type form of the *CHOP* AARE as shown by the presence of an ATF-2-supershifted DNA·protein complex (*lane 3*). If the mt4-containing oligonucleotide was used as a probe, anti-ATF-2 antibody supershifted the AARE mt4-bound complex (*lane 6*). In the case of mt15 and mt27, which showed no response to the amino acid availability, no ATF-2-supershifted DNA·protein complex was observed (*lanes 9* and *12*). Taken together, these results demonstrate that the nucleotides in the *CHOP* AARE core sequence required to confer amino acid responsiveness are also essential for the binding of ATF-2.

The AS Promoter Region (nt −68 to −60) Shares Structural and Functional Similarities with the CHOP AARE—The promoter of the human *AS* gene has been shown to contain two *cis*-elements, NSRE-1 and NSRE-2, which are both required to mediate the transcriptional activation of the gene in response to amino acid starvation (9). Sequence analysis of NSRE-1 (5′-GTTTCATCA-3′ on the bottom strand) revealed that it contains a sequence with high similarity to the 9-bp *CHOP* core consensus sequence (5′-(R/C)TT(R/T)CRTCA-3′) described above (Fig. 3*A*). Indeed the *AS* promoter sequence on the bottom strand (nt −57 to −75) revealed a high identity to the *CHOP* AARE on the top strand (nt −313 to −295), not only in the AARE core, but also in both of the border sequences. By nucleotide substitutions, this *AS* sequence had been shown by independent laboratories to be important for the response to amino acid starvation (8, 9). Furthermore, previous studies had shown that the *CHOP* AARE-like NSRE-1 sequence is not

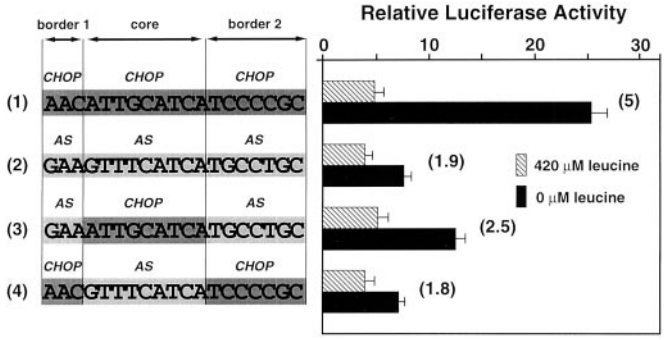

FIG. 4. **Role of the core and border sequences in the *CHOP* AARE and *AS* NSRE-1.** Oligonucleotides encoding the *CHOP* AARE, *AS* NSRE-1, or a chimeric AARE/NSRE-1 in which the 9-bp core sequence of the *CHOP* AARE and *AS* NSRE-1 were exchanged were inserted 5′ to the *TK* promoter in luciferase constructs. HeLa cells were transfected, incubated for 16 h in DMEM/F-12 with and without 420 μM leucine, and then harvested for preparation of cell extracts and luciferase activity determination. Relative luciferase activities were determined as described under "Materials and Methods." The relative -fold induction, defined as the ratio of the relative luciferase activity of leucine-starved cells to that of non-starved cells, is indicated in *parentheses*. Each data point represents the mean of at least three independent experiments performed in triplicate.

functional in the absence of NSRE-2 (9). To determine whether there are any circumstances under which NSRE-1 can, by itself, render a heterologous promoter amino acid-responsive, synthetic *AS* sequence oligonucleotides (nt −57 to −75) were inserted as one, two, or five copies immediately upstream of the minimum *TK* promoter (Fig. 3*B*). One or two copies of the *AS* sequence caused a very slight increase in transcription following amino acid deprivation (*rows 5* and *6*), whereas one or two copies of the *CHOP* AARE were sufficient to induce the luciferase activity (5- and 14-fold, respectively) in the absence of leucine (*rows 2* and *3*). However, five copies of the 19-bp *AS* promoter region (*row 7*) produced about the same strong amino acid response as obtained with five copies of the *CHOP* AARE (*row 4*). Therefore, we conclude that multiple copies of the *AS* NSRE-1 sequence can render a basal promoter amino acid-responsive and that it can be considered as an AARE. However, the *CHOP* AARE is notably more sensitive to amino acid starvation than is *AS* NSRE-1 alone. To determine whether it is the number of the NSRE-1 copies (five copies) or the distance of the fifth copy from the remainder of the promoter, three or four copies were mutated to leave one or two functional copies of the NSRE-1 sequence at a length equal to the construct containing five functional copies (*rows 8* and *9*). The data illustrate that having one or two functional copies of the NSRE-1 sequence at positions 4 and 5 did not result in activated transcription. Therefore, only when NSRE-1 is present in at least three copies does it have AARE-like capability. Moreover, kinetic analysis of luciferase activity driven by five copies of either the *CHOP* AARE or *AS* NSRE-1 revealed that induction of the activity was detectable 8 h after starvation and that a maximum induction level was reached after 16 h (data not shown). Taken together, these results show that five copies of the *CHOP* AARE and the *AS* NSRE-1 share functional similarities in response to leucine starvation.

Role of the Core and Border Sequences in the CHOP AARE and AS NSRE-1—To examine the respective role of the 9-bp AARE core and border sequences, chimeric AAREs were constructed in which the core sequences of the *CHOP* AARE and *AS* NSRE-1 were exchanged (Fig. 4). These 19-bp chimeric AAREs were inserted in a single copy upstream of the *TK* promoter. The constructs were transiently transfected into HeLa cells, and the response to leucine deprivation was determined by luciferase assay. Fusion of the *AS* border 1 and 2 sequences to the *CHOP* core sequence caused a decrease in the amino acid inducibility (compare *rows 1* and *3*), whereas the *CHOP* border sequences did not increase the amino acid responsiveness of a single copy of the *AS* core (compare *rows 2* and *3*). These data demonstrate that only the *CHOP* border sequences play a role in achieving the amino acid induction of the *CHOP* AARE.

Lack of ATF-2 Reduces (but Does Not Abolish) the Transcriptional Activation of AS by Leucine Starvation—It has been shown previously that ATF-2 has a critical role in the transcriptional activation of *CHOP* by leucine starvation (25). To determine the role of ATF-2 in the induction of *AS* expression, the effect of leucine starvation on *AS* mRNA expression was measured in MEFs deficient in ATF-2 and in the corresponding wild-type cells. Fig. 5*A* shows that like *CHOP*, *AS* exhibited a normal response to leucine starvation (4-fold increase) and to an agent (tunicamycin) that induces ER stress (33) in ATF-$2^{+/+}$ cells (5-fold increase). Lack of ATF-2 (ATF-$2^{-/-}$) resulted in a strong decrease in the *AS* mRNA inducibility (2-fold), whereas the amino acid inducibility of *CHOP* mRNA was completely lost. On the other hand, the induction of either *AS* or *CHOP* mRNA by the ERSR (tunicamycin) was not severely affected in ATF-$2^{-/-}$ cells (4-fold increase).

To more directly compare the effects of ATF-2 on regulation of *AS* and *CHOP* transcription by leucine deprivation, luciferase constructs containing the 3.4-kb *AS* 5′-upstream region (Fig. 5*B*, *rows 1* and *5*), the *CHOP* promoter (nt −649 to +91) (*rows 3* and *7*), two copies of *AS* NSRE-1 (*rows 2* and *6*), or one copy of the *CHOP* AARE (*rows 4* and *8*) inserted immediately upstream of the *TK* promoter were transiently transfected into ATF-2-deficient cells or into the corresponding wild-type MEF cells. The response to leucine was determined by luciferase assay under starved and non-starved conditions. Lack of ATF-2 caused a decrease in the amino acid inducibility of the *AS*-luciferase constructs (compare *rows 1* and *5* and *rows 2* and *6*), whereas it abolished the amino acid responsiveness of the *CHOP*-luciferase constructs (compare *rows 3* and *7* and *rows 4* and *8*). Taken together, these results provide evidence that in MEF cells, ATF-2 is not essential in the specific amino acid pathway that leads to the induction of *AS* transcription, but that ATF-2 is necessary to obtain the full *AS* response to amino acid starvation and is absolutely required for *CHOP* induction.

Difference between the CHOP AARE and AS NSRE in the Response to ER Stress—The *CHOP* and *AS* genes are transcriptionally regulated by amino acid starvation and the ERSR (15, 34). Fig. 6*A* shows that *CHOP* and *AS* gene expression was highly induced by leucine and by agents that induce ER stress such as tunicamycin. Barbosa-Tessmann *et al.* (9) have recently shown that activation of *AS* transcription by the amino acid response and ERSR occurs through the same common genomic elements. To determine whether the *CHOP* AARE is also involved in the ER stress pathway, the inducibility via this sequence by tunicamycin was tested. The results show that at a low tunicamycin concentration (0.25 μg/ml), but one that induced *AS* and *CHOP* genes in intact cells (Fig. 6*A*), transcription via the *CHOP* AARE did not increase, whereas the *AS* NSRE was responsive (Fig. 6*B*). A modest induction (2-fold) of luciferase activity through the *CHOP* AARE was detected only with very high tunicamycin concentrations. These observations demonstrate that the *CHOP* sequence (nt −313 to −295) functions more effectively as an AARE and confirm that the *AS* NSRE genomic sequence (nt −75 to −34) can mediate the response to both amino acid and ER stress pathways.

Role of the AS NSRE-2 Sequence in Mediating The ERSR—Given the sequence similarity of the *CHOP* AARE and *AS*

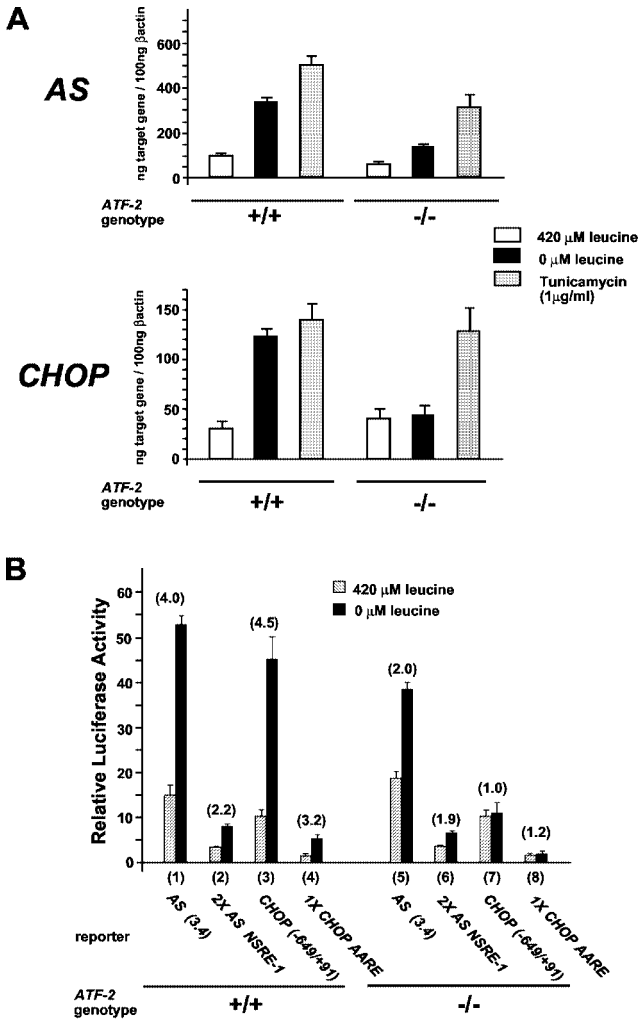

FIG. 5. **Lack of ATF-2 reduces (but does not abolish) the transcriptional activation of *AS* by leucine starvation.** *A*, wild-type (+/+) and mutant (−/−) ATF-2 MEFs were incubated for 16 h in DMEM/F-12 with and without 420 μM leucine or for 6 h in medium containing 1 μg/ml tunicamycin. Total RNA was extracted, and real-time reverse transcription-PCR was performed as described under "Materials and Methods." *B*, wild-type (+/+) and mutant (−/−) ATF-2 MEFs were transfected with luciferase reporter constructs containing the 3.4-kb *AS* 5′-upstream region (*AS (3.4)*), the *CHOP* promoter region from nt −649 to +91, two copies of the *AS* AARE (*2X AS NSRE-1*), or one copy of the *CHOP* AARE (*1X CHOP AARE*) inserted 5′ to the *TK* promoter. Twenty-four hours after transfection, cells were incubated for 16 h in DMEM/F-12 with and without 420 μM leucine and then harvested for preparation of cell extracts and determination of luciferase activity. Relative luciferase activities were determined as described under "Materials and Methods." The relative -fold induction, defined as the ratio of the relative luciferase activity of leucine-starved cells to that of non-starved cells, is indicated in *parentheses* above the bars.

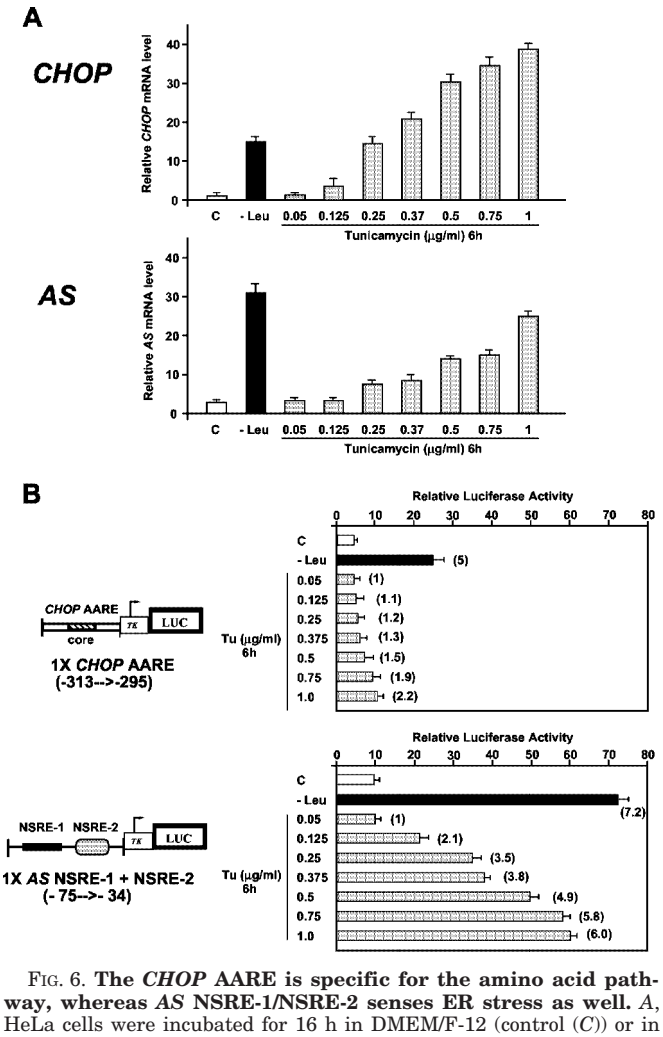

FIG. 6. **The *CHOP* AARE is specific for the amino acid pathway, whereas *AS* NSRE-1/NSRE-2 senses ER stress as well.** *A*, HeLa cells were incubated for 16 h in DMEM/F-12 (control (*C*)) or in DMEM/F-12 lacking leucine (−*Leu*) or for 6 h in DMEM-F12 containing different concentrations of tunicamycin (*Tu*; 0.05, 0.125, 0.25, 0.375, 0.5, 0.75, and 1 μg/ml) as indicated. Total RNA was extracted, and Northern blot analysis was performed. The blots were hybridized with human probes corresponding to *AS*, *CHOP*, or glyceraldehyde-3-phosphate dehydrogenase. Relative *CHOP* or *AS* mRNA levels were determined as the ratio of *CHOP* or *AS* mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA. *B*, HeLa cells were transfected with luciferase (*LUC*) constructs containing one copy of the *CHOP* AARE or *AS* NSRE-1/NSRE-2 (nt −75 to −34) inserted 5′ to the *TK* promoter and incubated under the conditions described for *A*. Relative luciferase activities were determined as described under "Materials and Methods." The relative -fold induction, defined as the ratio of the relative luciferase activity of leucine-starved cells to that of non-starved cells, is indicated in *parentheses* to the right of the bars. Each data point represents the mean of at least three independent experiments performed in triplicate.

NSRE-1 sequences, the possible decisive role of the *AS* NSRE-2 sequence in permitting the *AS* response to ER stress was investigated (Fig. 7*A*). As reported previously (9), mutation of the NSRE-2 sequence in the *AS* promoter resulted in a significant loss of induction by either amino acid deprivation or ER stress (*row 2*). Likewise, a functional NSRE-2 without the NSRE-1 sequence did not permit induction (*row 3*). As described above, the *CHOP* AARE alone did not mediate a response to ER stress (*row 4*). However, if the *AS* NSRE-2 sequence was placed downstream of the *CHOP* AARE, in a configuration similar to its physical relationship to NSRE-1 in the *AS* promoter, ER responsiveness was conferred to the *CHOP* AARE (*row 5*). Also noteworthy is the NSRE-2-mediated enhancement of the *CHOP* AARE response to leucine deprivation (5- *versus* 11.5-fold). Thus, the NSRE-2 sequence conveys both ER stress responsiveness and increased sensitivity to amino acid limitation.

Another difference between the *AS* and *CHOP* promoters is the presence of three upstream GC boxes (I–III) in the *AS* proximal promoter (9). It has recently been demonstrated that these three GC-rich sequences bind either Sp1 or Sp3, the former supporting basal *AS* transcription and the latter supporting both basal and stimulus-induced transcription (35). At least one GC box is required for maximum transcription from the *AS* gene. To determine whether the function of the *CHOP* AARE can be enhanced by the presence of a GC box, the NSRE-1 sequence was replaced with the *CHOP* AARE within the *AS* promoter (Fig. 7*B*). The presence of either the NSRE-2 sequence (compare *rows 3* and *4*) or the GC box III sequence

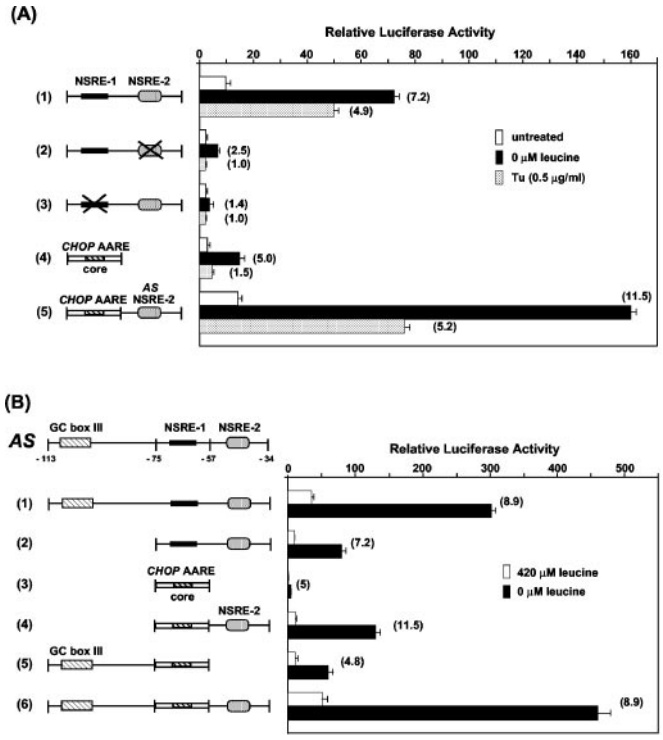

FIG. 7. **Effect of *AS* NSRE-2 and GC-rich sequences on *CHOP* AARE activity.** *A*, HeLa cells were transfected with luciferase constructs containing one copy of wild-type or mutant *AS* NSRE-1/NSRE-2 (nt −75 to −34), the *CHOP* AARE (nt −313 to −295), or a fusion of the *CHOP* AARE with *AS* NSRE-2 inserted 5′ to the *TK* promoter. Twenty-four hours after transfection, cells were incubated for 16 h in DMEM/F-12 with and without leucine or for 6 h in DMEM-F12 containing 0.5 μg/ml tunicamycin (*Tu*) and harvested for luciferase activity determination after the indicated incubation times. *B*, HeLa cells were transfected with luciferase constructs containing deletion and mutation series in the *AS* promoter, the *CHOP* AARE (nt −313 to −295), or a fusion of the *CHOP* AARE or the core sequence of the *CHOP* AARE (*boxed*; nt −310 to −302) with *AS* NSRE-2 or GC box III inserted 5′ to the *TK* promoter. Twenty-four hours after transfection, cells were incubated for 16 h in DMEM/F-12 with and without 420 μM leucine. Relative luciferase activities were determined as described under "Materials and Methods." The relative -fold induction, defined as the ratio of the relative luciferase activity of leucine-starved cells to that of non-starved cells, is indicated. Each data point represents the mean of at least three independent experiments performed in triplicate.

(compare *rows 3* and *5*) enhanced the basal transcription from the *CHOP* AARE alone, but the GC box did not increase the -fold induction after leucine deprivation either without the NSRE-2 sequence (compare *rows 3* and *5*) or with the NSRE-2 element (compare *rows 4* and *6*).

DISCUSSION

The human *CHOP* gene 5′-flanking region contains an AARE (nt −313 to −295) that is required for increasing transcription of the gene following amino acid deprivation. Our single-nucleotide mutagenesis affecting the 9-bp core sequence (nt −310 to −302) demonstrated that the T residues at positions 2, 3, and 7; the C at position 5; and the A at position 9 are required to confer amino acid responsiveness and established that the minimum core consensus sequence is 5′-(R/C)TT(R/T)CRTCA-3′. In the human *AS* gene, NSRE-1 is one of the two *cis*-elements (the other being NSRE-2) required for increased transcription following amino acid deprivation. NSRE-1 differs from the 9-bp *CHOP* core consensus sequence by only two nucleotides (the G at position 1 and the T at position 4), which seem not to be critical for the sensitivity to amino acid limitation. Barbosa-Tessmann *et al.* (9) have shown that the transcriptional activation of *AS* in response to amino acid starvation requires the presence of both NSRE-1 and NSRE-2. We have demonstrated here that multiple copies of NSRE-1 alone can functionally synergize with itself and confer amino acid responsiveness, whereas one copy of the *CHOP* AARE is sufficient to render a heterologous promoter amino acid-responsive. However, although the 9-bp *CHOP* core consensus sequence has an intrinsic ability to mediate the amino acid response, a functional AARE cannot be restricted to this sequence. Previous studies have documented, for example, that the ATF-binding site in the adenovirus E4 promoter (36, 37) includes this consensus sequence, but is not able to mediate amino acid inducibility (see Fig. 5*B* of Ref. 25). It became evident that the amino acid response is mediated by a set of *cis*-elements rather than by a single 9-bp element. Accessory elements, which have no intrinsic ability to mediate the amino acid response by themselves, may boost the -fold responsiveness mediated by the AARE core. Our results provide evidence that *AS* NSRE-2 can be considered as an accessory element because it is not able, by itself, to mediate amino acid inducibility, but enhances the response to amino acid limitation. In addition, when placed downstream of the *CHOP* AARE, NSRE-2 confers responsiveness to ER stress. However, the region immediately following the *CHOP* AARE does not have a readily identifiable sequence that corresponds to NSRE-2. We also documented that the border sequences of the *CHOP* AARE core appear to contain some accessory elements, whereas the border sequences of *AS* NSRE-1 do not.

Sequences of the *CHOP* AARE and *AS* NSRE-1 show some homology to the specific binding sites of the C/EBP and ATF/cAMP response element-binding protein transcription factors. We have previously demonstrated by electrophoretic mobility shift assay studies that the transcription factor ATF-2 and C/EBPβ bind the *CHOP* AARE under starved and non-starved conditions (25). Our present data demonstrate that the nucleotides in the 9-bp *CHOP* AARE core sequence required to confer amino acid responsiveness are also essential for the binding of ATF-2. When knockout cell lines for ATF-2 were tested, amino acid-dependent expression of *CHOP* was blocked, demonstrating that ATF-2 is essential for the transcriptional activation of *CHOP* by leucine starvation (25). This result was supported by the observation that expression of a dominant-negative form of ATF-2 suppressed the starvation-dependent transcription from a *CHOP* promoter-luciferase reporter construct. In contrast, Siu *et al.* (10) have demonstrated by electrophoretic mobility shift assay experiments that ATF-2 does not bind to the NSRE-1 sequence. Our present studies provide evidence that ATF-2 is necessary to obtain the full *AS* response. However, in cells devoid of ATF-2 expression, *AS* expression remains slightly inducible following amino acid starvation. We hypothesized that ATF-2 could activate gene transcription either by interactions with the transcriptional machinery or by direct effects on a chromatin component (38). Using electrophoretic mobility shift assay experiments, Siu *et al.* (10, 11) have recently demonstrated that both C/EBPβ and ATF-4 bind to NSRE-1 and that the amount of the C/EBPβ and ATF-4 complexes increased when extracts from amino acid-deprived cells were tested. Furthermore, expression of dominant-negative mutants of C/EBPβ (10) or ATF-4 (11) block amino acid-regulated transcription. In contrast, using knockout cell lines for C/EBPβ, we have previously demonstrated that C/EBPβ is not essential for the transcriptional activation of *CHOP* in response to amino acid deprivation, although this factor binds *in vitro* to the *CHOP* AARE (25). However, the binding of ATF-4 to the *CHOP* AARE and the possible role of this transcription factor in *CHOP* amino acid-regulated transcription remain to be demonstrated.

CHOP and *AS* are also transcriptionally regulated by the ERSR (15, 34). The ERSR (also known as the unfolded protein response) is an intracellular signaling pathway to remedy the accumulation of unfolded protein in the ER (39). Transcriptional control of *CHOP* by ER stress involves the binding of ATF-6 in the presence of NF-Y, TFII-I, and YY1 to the *cis*-acting ERSR element (ERSE) located between nt −75 and −93 (40, 41). The *CHOP* AARE does not have similarity to the ERSE consensus sequence, which is 5′-CCAATN$_9$CCACG-3′ (40). Consistent with this observation, we have demonstrated that the *CHOP* AARE cannot mediate the response by the ERSR pathway. In the case of *AS*, the promoter region lacks the ERSE consensus sequence. However, the combination of NSRE-1 and NSRE-2 mediates increased transcription following activation of the ER pathway (9). Because the present data document that NSRE-2 can confer ER stress responsiveness to the *CHOP* AARE, NSRE-2 appears to play a specific role in sensing ER stress. However, *AS* NSRE-2 does not have similarity to the ERSE consensus sequence. Therefore, the nucleotides essential for the transcriptional activity of NSRE-2 and the identity of the transcription factors that bind to it merit further investigation.

Collectively, from the results presented in this study, the *CHOP* AARE and *AS* NSRE-1 sequences have some structural and functional similarities. However, there are several lines of evidence suggesting that there are differences in the molecular mechanisms involved in the induction of *CHOP* and *AS* following amino acid starvation. 1) The *CHOP* AARE can function independently to some extent, whereas *AS* NSRE-1 is functionally weak by itself and instead requires the presence of NSRE-2 within a complex nutrient-sensing response unit. 2) The *cis*-acting elements required for induction of the *CHOP* gene following amino acid starvation (AARE) or the ERSR pathway (ERSE) are located in sequences separated by several hundred base pairs, whereas the *AS* NSRE-1 and NSRE-2 sequences required for activation of the gene following either amino acid limitation or activation of the ERSR pathway are separated by 11 bp (9). 3) ATF-2 binds *in vitro* to the *CHOP* AARE sequence and is essential for the transcriptional activation of *CHOP* by leucine starvation, whereas this transcription factor does not bind *in vitro* to the *AS* NSRE-1 sequence, but appears to be necessary to obtain the full *AS* response. 4) We have previously shown that the amino acid specificity with regard to the degree of induction of *CHOP* and *AS* is different (23).

The molecular mechanisms involved in the cellular response to amino acid availability have just begun to be discovered. By first identifying the genomic *cis*-elements and then the corresponding transcription factors responsible for regulation of specific target genes, it is anticipated that one can progress backwards up the signal transduction pathway to understand the individual steps required. The identification of different key transcriptional regulators for *CHOP* (ATF-2) and for *AS* (C/EBPβ and ATF-4) suggests that, as described in yeast, at least two independent pathways could lead to induced gene transcription in mammals. Defining the precise cascade of molecular events by which the cellular concentration of an individual amino acid regulates gene expression will be an important contribution to our understanding of metabolite control in mammalian cells. These studies will provide insight into the role of amino acids in the regulation of cellular functions such as cell division, protein synthesis, and proteolysis.

REFERENCES

1. Hinnebusch, A. G. (1994) *Trends Biochem. Sci.* **19,** 409–414
2. Hinnebusch, A. G. (1997) *J. Biol. Chem.* **272,** 21661–21664
3. Fafournoux, P., Bruhat, A., and Jousse, C. (2000) *Biochem. J.* **351,** 1–12
4. Laine, R. O., Hutson, R. G., and Kilberg, M. S. (1996) *Prog. Nucleic Acid Res. Mol. Biol.* **53,** 219–248
5. Gong, S. S., Guerrini, L., and Basilico, C. (1991) *Mol. Cell. Biol.* **11,** 6059–6066
6. Hutson, R. G., and Kilberg, M. S. (1994) *Biochem. J.* **303,** 745–750
7. Jousse, C., Bruhat, A., Ferrara, M., and Fafournoux, P. (2000) *J. Nutr.* **130,** 1555–1560
8. Guerrini, L., Gong, S. S., Mangasarian, K., and Basilico, C. (1993) *Mol. Cell. Biol.* **13,** 3202–3212
9. Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Siu, F., Schuster, S. M., Nick, H. S., and Kilberg, M. S. (2000) *J. Biol. Chem.* **275,** 26976–26985
10. Siu, F., Chen, C., Zhong, C., and Kilberg, M. S. (2001) *J. Biol. Chem.* **276,** 48100–48107
11. Siu, F., Bain, P. J., LeBlanc-Chaffin, R., Chen, H., and Kilberg, M. S. (2002) *J. Biol. Chem.* **277,** 24120–24127
12. Luethy, J. D., and Holbrook, N. J. (1992) *Cancer Res.* **52,** 5–10
13. Sylvester, S. L., Rhys, C. M. J., Luethy-Martindale, J., and Holbrook, N. J. (1994) *J. Biol. Chem.* **269,** 20119–20125
14. Schmitt-Ney, M., and Habener, J. F. (2000) *J. Biol. Chem.* **275,** 40839–40845
15. Wang, X.-Z., Lawson, B., Brewer, J. W., Zinszner, H., Sanjay, A., Mi, L.-J., Boorstein, R., Kreibich, G., Hendershot, L. M., and Ron, D. (1996) *Mol. Cell. Biol.* **16,** 4273–4280
16. Ron, D., and Habener, J. F. (1992) *Genes Dev.* **6,** 439–453
17. McKnight, S., Lane, M. D., and Gluecksohn-Waelsch, S. (1989) *Genes Dev.* **3,** 2021–2024
18. Roesler, W. J. (2001) *Annu. Rev. Nutr.* **21,** 141–165
19. Batchvarova, N., Wang, X.-Z., and Ron, D. (1995) *EMBO J.* **14,** 4654–4661
20. Fawcett, T. W., Eastman, H. B., Martindale, J. L., and Holbrook, N. J. (1996) *J. Biol. Chem.* **271,** 14285–14289
21. Ubeda, M., Wang, X.-Z., Zinszner, H., Wu, I., Habener, J. F., and Ron, D. (1996) *Mol. Cell. Biol.* **16,** 1479–1489
22. Sok, J., Wang, X.-Z., Batchvarova, N., Kuroda, M., Harding, H., and Ron, D. (1999) *Mol. Cell. Biol.* **19,** 495–504
23. Jousse, C., Bruhat, A., Hardind, H. P., Ferrara, M., Ron, D., and Fafournoux, P. (1999) *FEBS Lett.* **448,** 211–216
24. Bruhat, A., Jousse, C., Wang, X.-Z., Ron, D., Ferrara, M., and Fafournoux, P. (1997) *J. Biol. Chem.* **272,** 17588–17593
25. Bruhat, A., Jousse, C., Carraro, V., Reimold, A., Ferrara, M., and Fafournoux, P. (2000) *Mol. Cell. Biol.* **20,** 7192–7204
26. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) *Mol. Cell* **6,** 1099–1108
27. Maekawa, T., Bernier, F., Sato, M., Nomura, S., Singh, M., Inoue, Y., Tokunaga, T., Imai, H., Yokoyama, M., Reimold, A. M., Glimcher, L., and Ishii, S. (1999) *J. Biol. Chem.* **274,** 17813–17819
28. Todaro, G. J., and Green, H. (1963) *J. Cell Biol.* **17,** 299–313
29. Hall, C. V., Jacob, P. E., Ringold, M., and Lee, F. (1983) *J. Mol. Appl. Genet.* **2,** 101–109
30. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., p. 7.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Park, J. S., Luethy, J. D., Wang, M. G., Fargnoli, J., Fornace, A. J., Jr., McBride, O. W., and Holbrook, N. J. (1992) *Gene* (*Amst.*) **116,** 259–267
33. Zinszner, H., Kuroda, M., Wang, X.-Z., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) *Genes Dev.* **12,** 982–995
34. Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Schuster, S. M., Nick, H. S., and Kilberg, M. S. (1999) *J. Biol. Chem.* **274,** 31139–31144
35. Leung-Pineda, V., and Kilberg, M. S. (2002) *J. Biol. Chem.* **277,** 16585–16591
36. Hurst, H. C., and Jones, N. C. (1987) *Genes Dev.* **1,** 1132–1146
37. Lin, Y. S., and Green, M. R. (1988) *Proc. Acad. Natl. Sci. U. S. A.* **85,** 3396–3400
38. Kawasaki, H., Schiltz, L., Chiu, R., Itakura, K., Taira, K., Nakatani, Y., and Yokoyama, K. K. (2000) *Nature* **405,** 195–200
39. Kaufman, R. J. (1999) *Genes Dev.* **13,** 1211–1233
40. Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2000) *Mol. Cell. Biol.* **20,** 6755–6767
41. Parker, R., Phan, T., Baumeister, P., Roy, B., Cheriyath, V., Roy, A. L., and Lee, A. S. (2001) *Mol. Cell. Biol.* **21,** 3220–3233