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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*

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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 ciselements 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/EBP β 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*

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¹ The abbreviations used are: AS, asparagine synthetase; C/EBP, CCAAT/enhancer-binding protein; CHOP, <u>C</u>/EBP <u>ho</u>mologous 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, Dulbeco'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 12well 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 μ g of pCMV- β Gal, 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 μ l 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 -Galacto-sidase 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-TATATK-LUC, containing the minimum herpes simplex virus promoter for thymidine kinase (TK; nt -40 to +50), was generated as previously described (25). Plasmids $1 \times -$, $2 \times -$, and 5×CHOPAARE-TATATK-LUC were constructed by inserting SstI-XhoI double-stranded oligonucleotides containing one, two, and five iterations of the CHOP AARE sequence, respectively, into the TATATK-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 TATATK-LUC plasmid, producing $1 \times -$, $2 \times -$, and $5 \times ASNSRE-1$ -TATATK-LUC, respectively. Mutation series in the CHOP AARE sequence and deletion and mutation series in the AS promoter were made by inserting SstI-XhoI double-stranded mutated sequences into the TATATK-LUC plasmid. pCHOP-LUC (nt -649 to +91) was generated as previously described (25). Plasmid p3.4AS-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 XhoI and HindIII 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, $6 \times$ SSC, $5 \times$ Denhardt's reagent, 0.5% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 10 µg/ml yeast tRNA. The human CHOP $\operatorname{cDNA}(\operatorname{BH1})$ 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 $[\alpha^{-32}P]dCTP$ (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 $2 \times$ SSC containing 0.1% SDS, $0.5 \times$ SSC containing 0.1% SDS, and $0.1 \times$ 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-3phosphate 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 ($0.5 \ \mu$ g) was reverse-transcribed with 100 units of Superscript II Plus RNase H⁻ reverse transcriptase (Invitrogen) using 100 μ M random hexamer primers (Amersham Biosciences) according to the manufacturer's instructions. Primers for *CHOP* (forward primer, 5'-ctagcttggctgacagage-3'; and reverse primer, 5'-ctgctcttctctctage-3') and AS (forward primer, 5'-tacaaccacaaggcgetaca-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'- tacagcttcaccaccacgc-3') and reverse $(5^\prime\text{-}aaggaaggctggaaaagagc-3^\prime)$ 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-Teasy 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 $^{\rm TM}$ 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 μ l of water, 1.6 μ l of MgCl₂ (3 mM), 1 μ l of forward primer (0.5 μ M), 1 μ l of reverse primer (0.5 μ M), 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 μ l 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'-ATTG-CATCA-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).

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. 2A). A major specific DNA-protein complex was



consensus R/cTTR/TCRTCA

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 $\mu{\rm M}$ 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 $(\mathbf{R} = \mathbf{G} \text{ or } \mathbf{A})$.

detected after incubation of non-starved HeLa nuclear extracts with the ³²P-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. \hat{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 μ l 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.

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. 2B). ATF-2 bound to the wildtype form of the CHOP AARE as shown by the presence of an ATF-2-supershifted DNA protein complex (lane 3). If the mt4containing 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.



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. \vec{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.

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. 3A). 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 μ M 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. 3B). One or two copies of the ASsequence 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 acidresponsive 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. 5A 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 $\text{ATF-}2^{-/-}$ 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. 5B, 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 ASluciferase 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. 6A 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 μ g/ml), but one that induced AS and CHOP genes in intact cells (Fig. 6A), transcription via the CHOP AARE did not increase, whereas the AS NSRE was responsive (Fig. 6B). 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 realtime 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 μ M 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.

NSRE-1 sequences, the possible decisive role of the AS NSRE-2 sequence in permitting the AS response to ER stress was investigated (Fig. 7A). 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 down-stream 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-



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.

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. 7B). 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. Twentyfour 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 μ g/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/C)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. 5B 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/EBP β 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 dominantnegative 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/EBP β and ATF-4 bind to NSRE-1 and that the amount of the C/EBP β and ATF-4 complexes increased when extracts from amino aciddeprived cells were tested. Furthermore, expression of dominant-negative mutants of C/EBP β (10) or ATF-4 (11) block amino acid-regulated transcription. In contrast, using knockout cell lines for C/EBP β , we have previously demonstrated that C/EBP β 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 cisacting 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'-CCAATN₉CCACG-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 cisacting 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/ $EBP\beta$ 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.

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