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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*)<sup>1</sup> 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/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 malformed proteins (15). *CHOP*

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<sup>1</sup> 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.



tacagcttcaccaccacagc-3') and reverse (5'-aaggaagctggaaaagagc-3') primers.

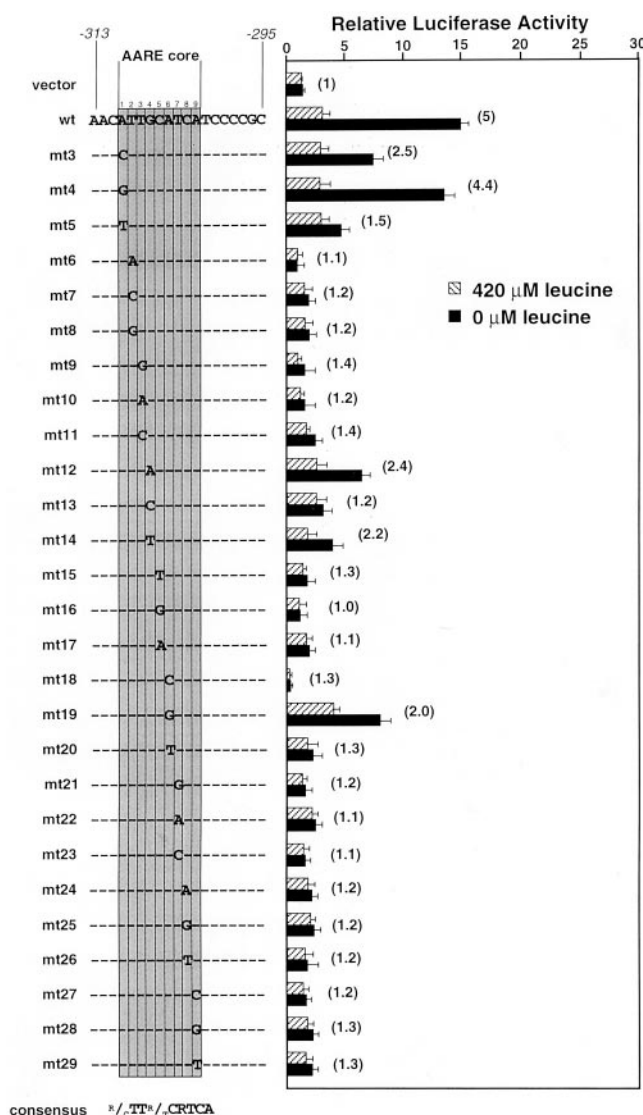
Quantification involved the use of standard curves that had been prepared with plasmids containing specific sequences of each gene. We cloned the PCR products of *CHOP*, *AS*, and  $\beta$ -actin into the pGEM-T-easy vector (Promega) according to the manufacturer's instructions. For the construction of standard curves for *CHOP*, *AS*, and  $\beta$ -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  $\mu$ l of water, 1.6  $\mu$ l of  $MgCl_2$  (3 mM), 1  $\mu$ l of forward primer (0.5  $\mu$ M), 1  $\mu$ l of reverse primer (0.5  $\mu$ M), and 2  $\mu$ l of LightCycler-FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals). The LightCycler master mixture (16  $\mu$ l) was filled in the LightCycler glass capillaries, and 4  $\mu$ 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  $\beta$ -actin. Each experiment was repeated three times to confirm the reproducibility of the results.

## RESULTS

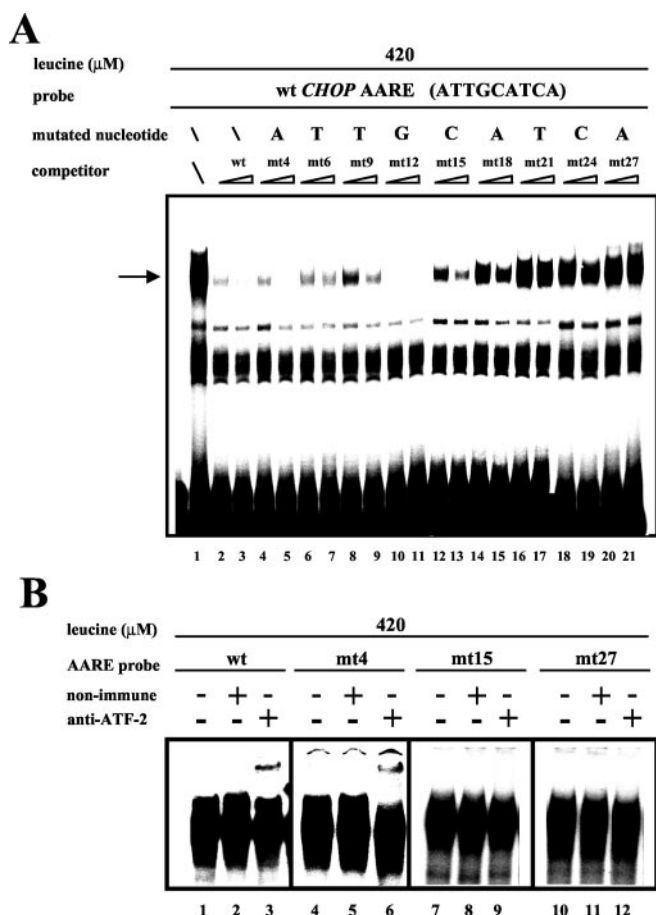
**The Nucleotides Required in the *CHOP* AARE Core Sequence to Mediate Transcriptional Induction by Leucine Are Essential for the Binding of ATF-2**—We have previously shown that the minimum core sequence in the *CHOP* AARE able to render a heterologous promoter amino acid responsive is 5'-ATTG-CATCA-3' (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



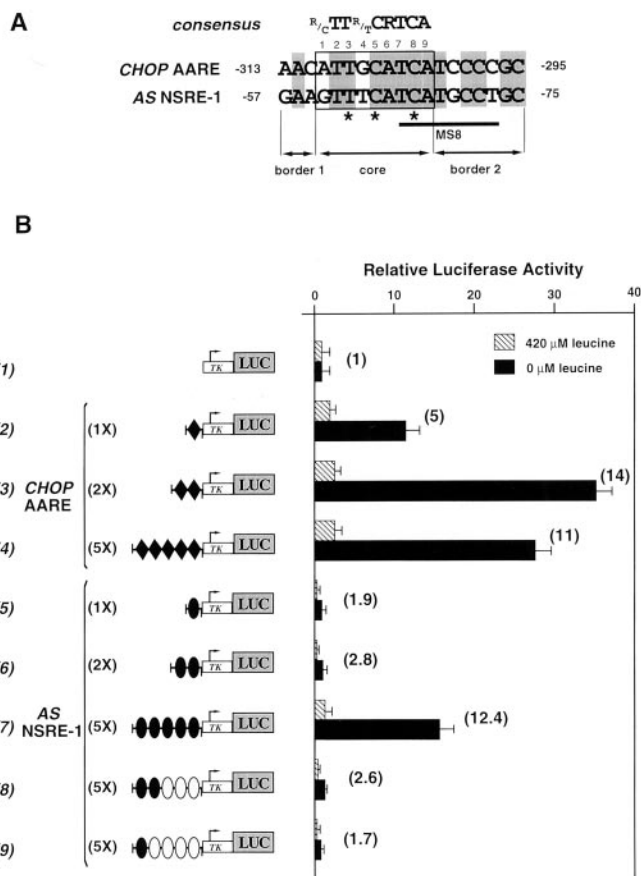
**FIG. 1. Identification of the nucleotides in the *CHOP* AARE core sequence required to mediate amino acid responsiveness.** HeLa cells were transfected with luciferase constructs containing a single copy of the native (wild-type (*wt*)) or mutant *CHOP* AARE (nt -313 to -295) inserted 5' to the *TK* 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  $\mu$ 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 (R = G or A).

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),



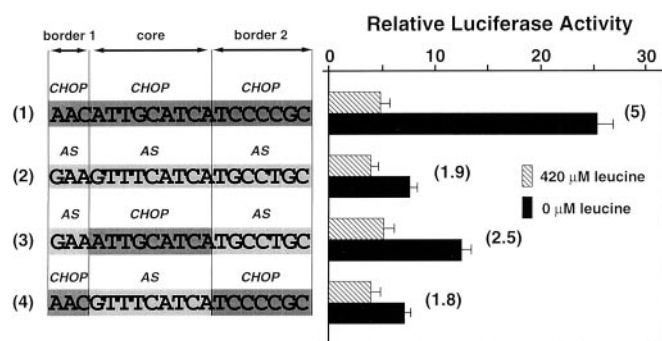
**FIG. 2. Nucleotides in the CHOP AARE core sequence required to confer amino acid responsiveness are essential for the binding of ATF-2.** *A*, gel mobility shift assays of nuclear extracts from HeLa cells incubated for 16 h in DMEM/F-12 containing 420  $\mu\text{M}$  leucine. The 19-bp wild-type CHOP 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  $\mu\text{l}$  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. 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.



**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 core consensus 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  $\mu\text{M}$  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 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. 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  $\mu$ 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 *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 deter-

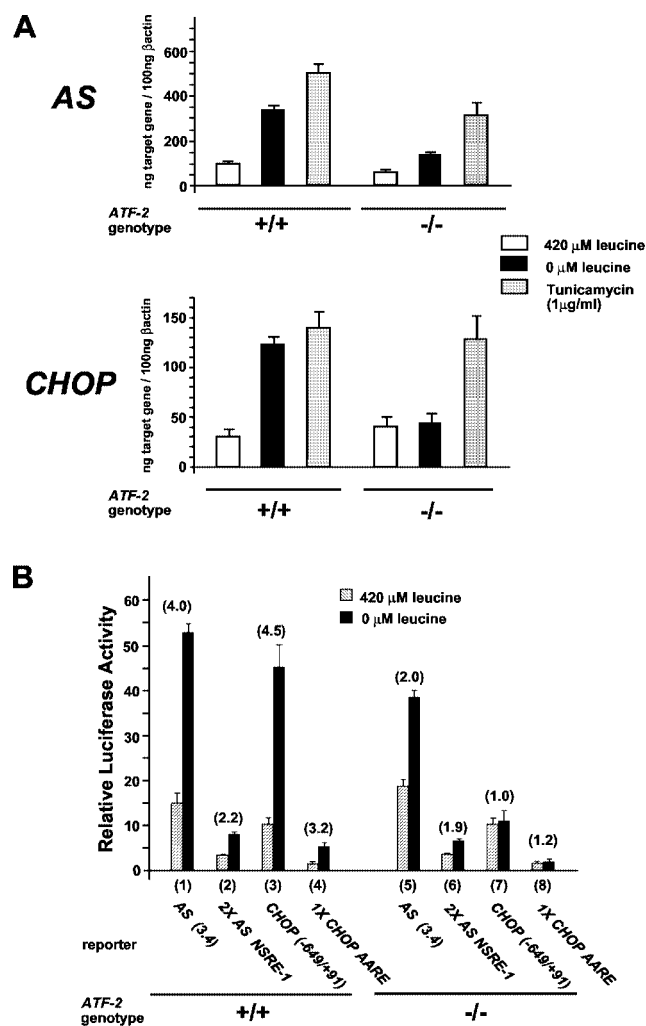
mined by luciferase assay. Fusion of the *AS* border 1 and 2 sequences to the *CHOP* core sequence caused a decrease in the amino acid inducibility (compare rows 1 and 3), whereas the *CHOP* border sequences did not increase the amino acid responsiveness of a single copy of the *AS* core (compare rows 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<sup>+/+</sup> cells (5-fold increase). Lack of ATF-2 (ATF-2<sup>-/-</sup>) 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<sup>-/-</sup> 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 *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.

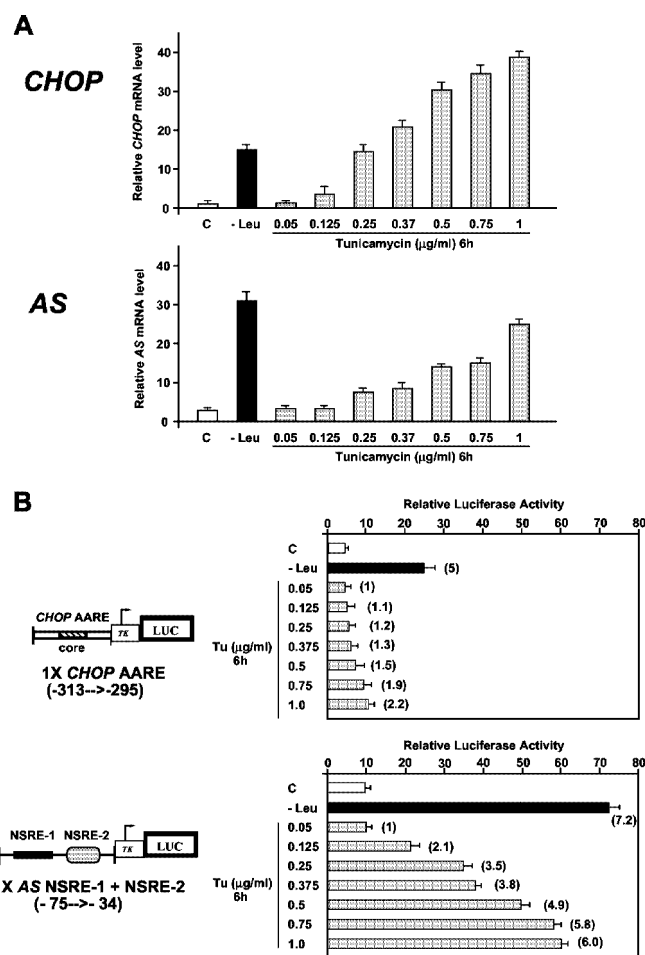
**Difference between the *CHOP* AARE and *AS* NSRE in the Response to ER Stress**—The *CHOP* and *AS* genes are transcriptionally regulated by amino acid starvation and the ERSR (15, 34). Fig. 6A shows that *CHOP* and *AS* gene expression was highly induced by leucine and by agents that induce ER stress 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  $\mu$ 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 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.

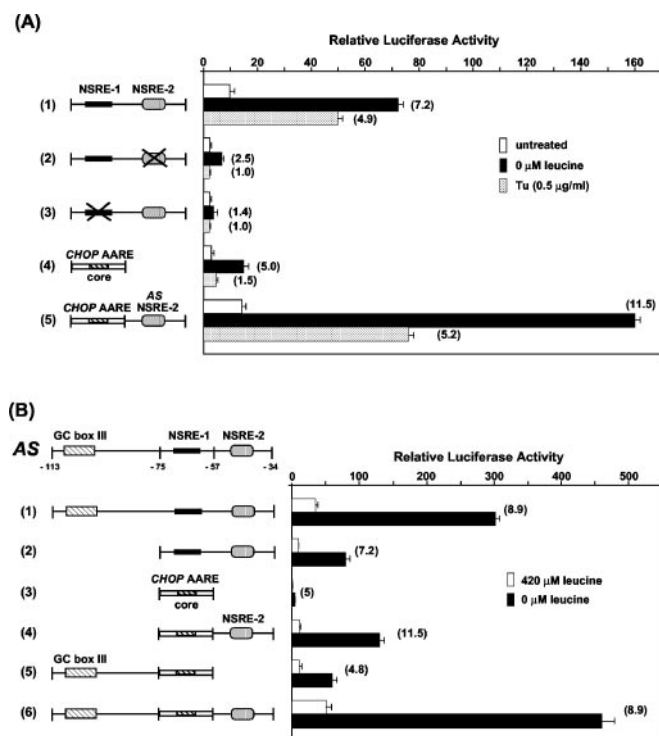
NSRE-1 sequences, the possible decisive role of the AS NSRE-2 sequence in permitting the AS response to ER stress was investigated (Fig. 7A). As reported previously (9), mutation of the NSRE-2 sequence in the AS promoter resulted in a significant loss of induction by either amino acid deprivation or 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 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-



**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. 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  $\mu$ 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  $\mu$ M 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 starva-

tion 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 $\beta$  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/EBP $\beta$  and ATF-4 bind to NSRE-1 and that the amount of the C/EBP $\beta$  and ATF-4 complexes increased when extracts from amino acid-deprived cells were tested. Furthermore, expression of dominant-negative mutants of C/EBP $\beta$  (10) or ATF-4 (11) block amino acid-regulated transcription. In contrast, using knockout cell lines for C/EBP $\beta$ , we have previously demonstrated that C/EBP $\beta$  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<sub>9</sub>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/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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