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Induction of *CHOP* Expression by Amino Acid Limitation Requires Both ATF4 Expression and ATF2 Phosphorylation*

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The *CHOP* gene is transcriptionally induced by amino acid starvation. We have previously identified a genomic *cis*-acting element (amino acid response element (AARE)) involved in the transcriptional activation of the human *CHOP* gene by leucine starvation and shown that it binds the activating transcription factor 2 (ATF2). The present study was designed to identify other transcription factors capable of binding to the *CHOP* AARE and to establish their role with regard to induction of the gene by amino acid deprivation. Electrophoretic mobility shift assay and transient transfection experiments show that several transcription factors that belong to the C/EBP or ATF families bind the AARE sequence and activate transcription. Among all these transcription factors, only ATF4 and ATF2 are involved in the amino acid control of *CHOP* expression. We show that inhibition of ATF2 or ATF4 expression impairs the transcriptional activation of *CHOP* by amino acid starvation. The transacting capacity of ATF4 depends on its expression level and that of ATF2 on its phosphorylation state. In response to leucine starvation, ATF4 expression and ATF2 phosphorylation are increased. However, induction of ATF4 expression by the endoplasmic reticulum stress pathway does not fully activate the AARE-dependent transcription. Taken together our results demonstrate that at least two pathways, one leading to ATF4 induction and one leading to ATF2 phosphorylation, are necessary to induce *CHOP* expression by amino acid starvation. This work was extended to the regulation of other amino acid regulated genes and suggests that ATF4 and ATF2 are key components of the amino acid control of gene expression.

All cells regulate gene expression in response to changes in the external environment such as nutrients availability. In mammals, plasma concentrations of nutrients are markedly affected by dietary or pathological conditions. The concentration of amino acids in the plasma is particularly sensitive to the nutritional state, with levels falling severalfold in cases of malnutrition. This may occur in response to a global limitation in protein intake or in response to specific limitations in essential amino acids (1–3).

The current understanding of mechanisms involved in amino acid-dependent control of gene transcription has just begun to be clarified in mammalian cells (4–6). At the molecular level, most of the results have been obtained by studying the transcriptional regulation of asparagine synthetase (*AS*)[1] and *CHOP* (C/EBP homologous protein, also called *GADD153*) 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 to amino acid starvation (7–9). Barbosa-Tessmann *et al.* (10) have identified, in the *AS* 5′-flanking region, two *cis*-elements termed nutrient-sensing response elements (NSRE-1, nt −68 to −60; NSRE-2, nt −48 to −43) that are essential for transcriptional activation by amino acid limitation. Electrophoretic mobility shift assay (EMSA) and overexpression of dominant negative mutants show that activation of the *AS* gene by amino acid limitation involves ATF-4 and C/EBPβ binding to the NSRE-1 site (11, 12).

The *CHOP* gene encodes a nuclear protein related to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors (13). 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 (14, 15). *CHOP* is induced by a wide variety of stresses and agents (16–18). The regulation of *CHOP* mRNA expression by amino acid concentration has both transcriptional and post-transcriptional components (19). An amino acid response element (AARE) was discovered between nucleotides −313 and −295 in the *CHOP* promoter. This element is sufficient to confer amino acid responsiveness to a heterologous promoter. The minimum core sequence (5′-ATTGCATCA-3′) is related to C/EBP and ATF/CRE binding sites and was described to bind *in vitro* the activating transcription factor 2 (ATF2) in starved and non-starved conditions. Using ATF2-deficient mouse embryonic fibroblasts, the expression of ATF2 was shown to be essential for the transcriptional activation of *CHOP* by leucine starvation (20).

Both *CHOP* and *AS* are also induced to high level during the endoplasmic reticulum (ER) stress response, itself presumably mediated by the accumulation of malfolded proteins (18). The *CHOP* promoter contains an ER stress response element (ERSE; nt −93 to −75), distinct from the AARE site, that

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[1] The abbreviations used are: *AS*, asparagine synthetase; CHOP, C/EBP homologous protein; NSRE, nutrient-sensing response element; ATF, activating transcription factor; AARE, amino acid response element; ER, endoplasmic reticulum; ERSE, ER stress response element; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; RT, reverse transcriptase; siRNA, small interference RNA; HAT, histone acetyl transferase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase.

mediates the activation by ER stress (21), whereas NSRE1 and NSRE2 of the *AS* gene are involved in the response to both amino acid starvation and ER stress (10).

To understand the individual steps required in the cellular response to amino acid limitation, our objective is to progress backwards up the signal transduction pathway from the amino acid response elements. The first step to achieve this goal is to identify transcription factors capable of binding to the AARE of the *CHOP* promoter and to establish their role with regard to induction of the gene by amino acid deprivation. Evidence is given here that several transcription factors that belong to the C/EBP or ATF families can bind the AARE sequence and activate the AARE-dependent transcription. However, only ATF4 and ATF2 are directly involved in the amino acid regulation of *CHOP* expression. Our results demonstrate that at least two pathways, one leading to ATF4 induction and one leading to ATF2 phosphorylation, are necessary to induce *CHOP* expression by amino acid starvation. This work was extended to the regulation of other amino acid-regulated genes and suggests that ATF4 and ATF2 are key components of the amino acid control of gene expression.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions—HeLa cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (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, dialyzed calf serum was used. Mouse embryonic fibroblasts deficient in ATF2 were kindly given by Dr. A. Reimold (University of Texas, Dallas).

DNA Transfection and Luciferase Assay—Cells were plated in 12-well dishes and transfected by the calcium phosphate coprecipitation method as described previously (19). Two micrograms of luciferase plasmid was 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. In the experiments using cDNA expression plasmid, a mixture containing 1 μg of luciferase plasmid, 1 μg of cDNA expression plasmid, and 0.05 μg of pCMV-βGal was transfected. In the control assay, the total amount of plasmid DNA was adjusted to 2 μg by addition of the plasmid lacking the cDNA to be expressed. Cells were then exposed to the precipitate for 16 h, washed twice in phosphate-buffered saline, and then incubated with DMEM/F-12 containing 10% calf serum. Twenty-four hours after transfection, cells were leucine-starved for 16 h then harvested in 100 μl of lysis buffer (Promega) and centrifuged at 13,000 $\times$ *g* for 2 min. 20 μl of the supernatant was assayed for luciferase activity (PRODEMAT, Anduze, France). β-Galactosidase activity was measured as described previously (22). Relative luciferase activity was given as the ratio of relative luciferase unit/relative β-galactosidase unit. 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 (20).

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.

Electrophoretic Mobility Shift Assays—Gel mobility shift assays were performed as described previously (20). For supershift analysis, 2 μg of specific antibody was added to the incubation mixture at room temperature 1 h prior to the addition of the labeled probe. Each mobility shift experiment was repeated three times to confirm the reproducibility of the results. The sequences of the probes were: *CHOP*-AARE (5′-aacattgcatcatccccgc-3′), ATF binding site (5′-atccggtgacgtcacggggg-3′) (23), and C/EBP binding site (5′-tgcagattgcgcaatctgca-3′) (24).

Plasmid Constructions—TATA*TK*-LUC containing the minimum herpes simplex virus promoter for thymidine kinase (−40 to +50) was generated as previously described (20). 1X *CHOP*AARE-*TK*-LUC plasmid was constructed by inserting SstI-XhoI double strand oligonucleotides containing one copy of the *CHOP* AARE sequence into *TK*-LUC plasmid. Mutation series in the *CHOP* AARE sequence were made by inserting SstI-XhoI double strand mutated sequences into the TATA*TK*-LUC plasmid. p*CHOP*-LUC (−954 to +91) was generated as previously described (20). All the luciferase plasmid constructs were sequenced before utilization using the ABI PRISM Bigdye terminator cycle sequencing reaction kit and the ABI PRISM 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

The expression plasmids for ATF1, ATF2, ATF3, ATF4, ATF7, C/EBPα, C/EBPβ, C/EBPδ, and C/EBPγ were generously provided by Drs. S. Ishii (Riken, Tsukuba Life Science Center, Japan), T. Hai (Ohio State University), B. Chatton (UM7100, ESBS, Illkirch, France), U. Schibler (Université de Genève, Swisserland), I. Lassot (Institut Cochin, Paris, France), and M. Li-weber (Cancer Research Center, Heidelberg, Germany). For each expression plasmid, a control plasmid was constructed by removing the cDNA.

Analysis of Gene Expression Using Real-time RT-PCR—Total RNA was prepared using a RNeasy mini kit (Qiagen) and treated with DNase I, Amp 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 human sequences (used in HeLa cell): *CHOP* (forward primer, 5′-cagaaccagcagaggtcaca-3′; reverse primer, 5′-agctgtgccactttcctttc-3′), *AS* (forward primer, 5′-atcactgtcgggatgtaccc-3′; reverse primer, 5′-cttcaacagagtggcagcaa-3′), ATF2 (forward primer, 5′-accatggtgcctagtgttcc-3′; reverse primer, 5′-gtggctggctgttgtaatga-3′), ATF3 (forward primer, 5′-gccattggagagctgtcttc-3′; reverse primer, 5′-gggccatctggaacataaga-3′), ATF7 (forward primer, 5′-ggatgagaaaaaggctgctg-3′; reverse primer, 5′-gacgtacaatggtgggtgtg-3′), C/EBPβ (forward primer, 5′-ctcgcaggtcaagagcaag-3′; reverse primer, 5′-gacagctgctccacctctct-3′), C/EBP8 (forward primer, 5′-ccgacctctttcaacagcaa-3′; reverse primer, 5′-caagctcaccacggtctgt-3′), SLC1A5 (forward primer, 5′-aagatcgtggagatggaggat-3′; reverse primer, 5′-gaactggaagaggtcccaaag-3′), SARS (forward primer, 5′-gaggacctgcccatcaagta-3′; reverse primer, 5′-tagaactcctctgcggtggt-3′), YARS (forward primer, 5′-gatcggaaggaggatgtgaa-3′; reverse primer, 5′-ttttccaggtccacgtaagc-3′), 4EBP1 (forward primer, 5′-tatgaccggaaattcctgatg-3′; reverse primer, 5′-ccatctcaaactgtgactcttca-3′), and 4F2 (forward primer, 5′-tcttgattgcggggactaac-3′; reverse primer, 5′-gccttgcctgagacaaactc-3′). Primers for mouse sequences (used in ATF2−/− and ATF2+/+ cells): *CHOP* (forward primer, 5′-cctagcttggctgacagagg-3′; reverse primer, 5′-ctgctccttctccttcatgc-3′), *AS* (forward primer, 5′-tacaaccacaaggcgctaca-3′; reverse primer, 5′-aagggcctgactccataggt-3′), ATF3 (forward primer, 5′-cgccatccagaataaacacc-3′; reverse primer, 5′-gcaggcactctgtcttctcc-3′), SLC1A5 (forward primer, 5′-tgctttcgggacctcttct-3′; reverse primer, 5′-cccgtttagttgtgcgatg-3′), SARS (forward primer, 5′-tcgatgacctcactgcagac-3′; reverse primer, 5′-ctttgttgtctgcgtcctca-3′), YARS (forward primer, 5′-ctgggttaactgggagcaaa-3′; reverse primer, 5′-tccaccccacttctcatctc-3′), 4EBP1 (forward primer, 5′-aactcacctgtggccaaaac-3′; reverse primer, 5′-ccatctcaaactgtgactcttca-3′), and 4F2 (forward primer, 5′-ttttggacctcactcccaac-3′; reverse primer, 5′-ctgccactcagccaagtaca-3′) were used and yielded PCR products 200 bp in size. To normalize for mRNA content, β-actin mRNA was also amplified: primers for the human β-actin sequence (forward primer, 5′-tccctggagaagagctacga-3′; reverse primer, 5′-agcactgtgttggcgtacag-3′) and primers for mouse β-actin sequence (forward primer, 5′-tacagcttcaccaccacagc-3′; reverse primer, 5′-aaggaaggctggaaaagagc-3′).

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 the studied genes and β actin into the pGEM-T easy vector (Promega) according to the manufacturer's instructions. For the construction of standard curves pGEM-T easy plasmids containing the amplified sequence were prepared as 10-fold serial dilution in water, from 4 ng to 0.4 pg.

PCR was carried out using a LightCycler™ System (Roche Applied Science), which allows amplification and detection (by fluorescence) in the same tube, using a kinetic approach. For LightCycler PCR reactions, a mastermix of the following reaction components was prepared to the indicated end concentration: 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-Fast Start DNA Master SYBR Green I (Roche Diagnostics). LightCycler mastermix (16 μl) was filled in the LightCycler glass capillaries, and 4 μl of cDNA (2 ng of reverse-transcribed total RNA) was 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 per second and a continuous fluorescence measurement), and finally a cooling step to 40 °C. A negative control without cDNA tem-

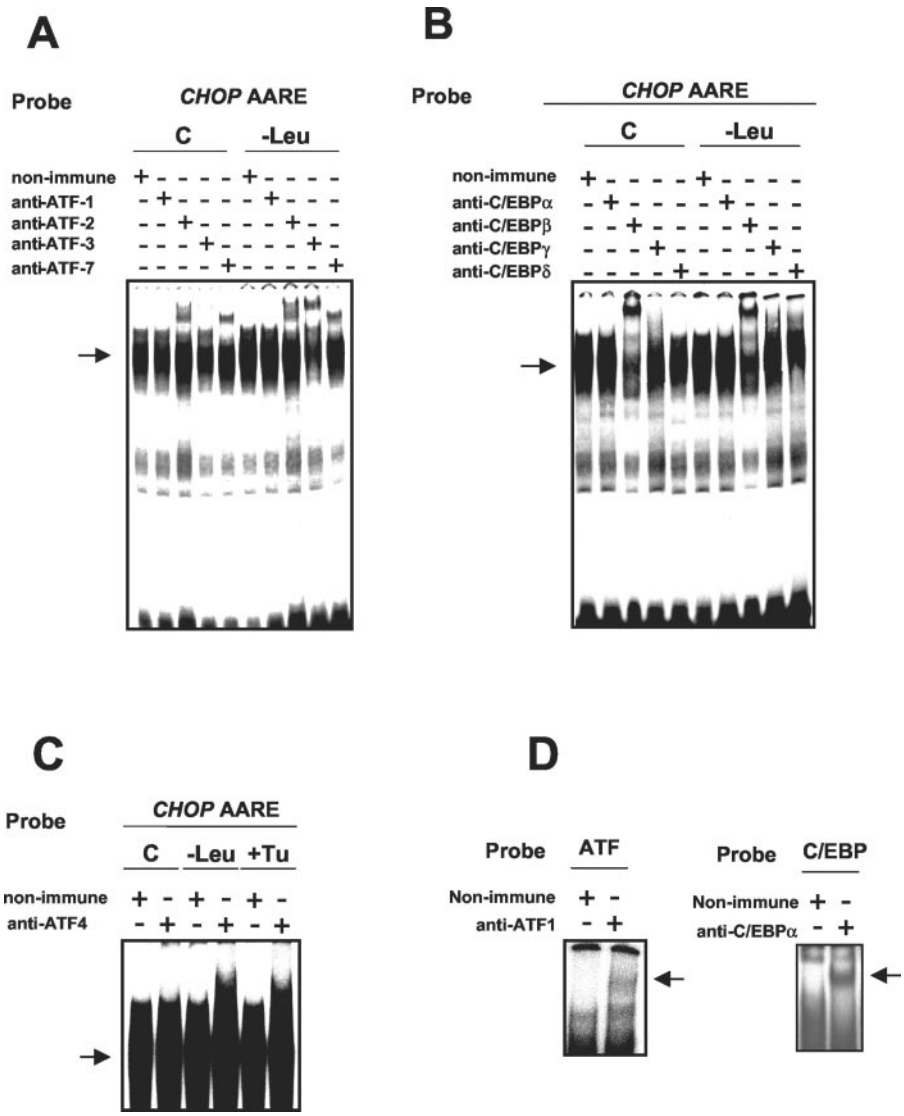

FIG. 1. **Transcription factors that belong to the C/EBP and ATF families bind the CHOP AARE sequence *in vitro*.** Electrophoretic mobility shift assay (EMSA) using antibodies against transcription factors that belong to the ATF (*A* and *C*) or C/EBP (*B*) families were performed as described under "Materials and Methods." Nuclear extracts were prepared from HeLa cells incubated for 8 h in DMEM/F-12 with or without (420 μM) leucine. In *C* we also used nuclear extracts from HeLa cells treated for 5 h with 5 μg/ml tunicamycin. The 19-bp *CHOP* AARE radiolabeled probes carried nucleotides −313 to −295. Nuclear extracts were first incubated with 1 μl of non-immune or immune serum, and the 19-bp *CHOP* AARE probes were then added as described under "Materials and Methods." In *D* super-shift experiment using nuclear extracts from fed cells in the presence of a probe encoding the consensus sequence of the ATF1 or C/EBPα binding sites (23, 24). Nuclear extracts from HepG2 cells (expressing large amount of C/EBPα protein) were used for testing the C/EBPα antibody.

plate 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 were displayed in nanograms of *CHOP* or *AS* per 100 ng of β-actin. Each experiment was repeated three times to confirm the reproducibility of the results.

siRNA Preparation and Transfection—siRNA corresponding to ATF4 (5′-gcguaguucgcuaaggugadTdT-3′ and 5′-ucaccuuagcgaacuacgcdTdT-3′) and ATF3 (5′-gcaccucugccaccggaugdTdT-3′ and 5′-cauccgguggcagaggugcdTdT-3′) mRNA were designed as recommended (25, 26). An siRNA corresponding to ATF4 siRNA but mutated on three nucleotides (5′-gcguaguuagcuaaggugadTdT-3′ and 5′-ucaccuuagcgaacuacgcdTdT-3′) was used as a negative control. Each pair has a 3′ overhang of 2 nucleotides on each side. Designed RNA oligonucleotides were blasted against the GenBank™/EMBL data base to ensure gene specificity. They were obtained from Eurogentec (Liège, Belgium). Annealing was performed as described by Elbashir (27): the complementary two strands (each 20 μM) in 200 μl of annealing buffer (100 mM potassium acetate; 30 mM HEPES/KOH, pH7.4; 2 mM magnesium acetate) were heated for 1 min at 90 °C and then incubated for 1 h at 37 °C.

One day before transfection with siRNA, HeLa cells were plated in 6-well plates at 25% confluency. Then, 2 μg of siRNA was introduced into the cells using the calcium phosphate precipitation as described above. The next day, cells were trypsinized and plated in 12-well plates. Transfection with reporter vectors was carried out 2 days after the siRNA transfection using calcium phosphate precipitation.

Immunoblot Analysis—Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 2 mM Na_3VO_4, 100 nM acid okadaic, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktails from Sigma), then proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature with a solution of 5% nonfat milk powder in TN (50 mM Tris-HCl, pH 8.0, 150 mM NaCl). The blots were then incubated with antibody in blocking solution overnight at 4 °C. Antibodies were diluted according to the manufacturer's instructions. The blots were washed four times in TN containing 0.05% Triton X-100 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000) Cell-Signaling Technology (7074#) in blocking buffer for 1 h at room temperature. After five washes, the blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Antibodies—Antibody 9221#S against Phospho-ATF2 Thr-71 was from Cell Signaling Technology (Beverly, MA). Antibodies against ATF1, ATF2, ATF3, ATF4, C/EBPα, C/EBPβ, and C/EBPδ were from Santa Cruz Biotechnology (respectively, 243X, 6233X, 188X, 200X, 61X, 7962X, and 151X). Anti-ATF7 and C/EBPγ antibodies were kindly given by Drs. B. Chatton (UM7100, ESBS, Illkirch, France), K. Calame (Columbia University, New York, NY), and P. Johnson (NCI, National Institutes of Health, Frederick, MD).

RESULTS

Transcription Factors That Belong to the C/EBP and ATF Families Bind the CHOP AARE Sequence in Vitro—We previously identified a *cis*-acting element (AARE) involved in the transcriptional activation of the human *CHOP* gene by leucine starvation and showed that it binds the activating transcription factor2 (ATF2). Computer analysis showed that other tran-

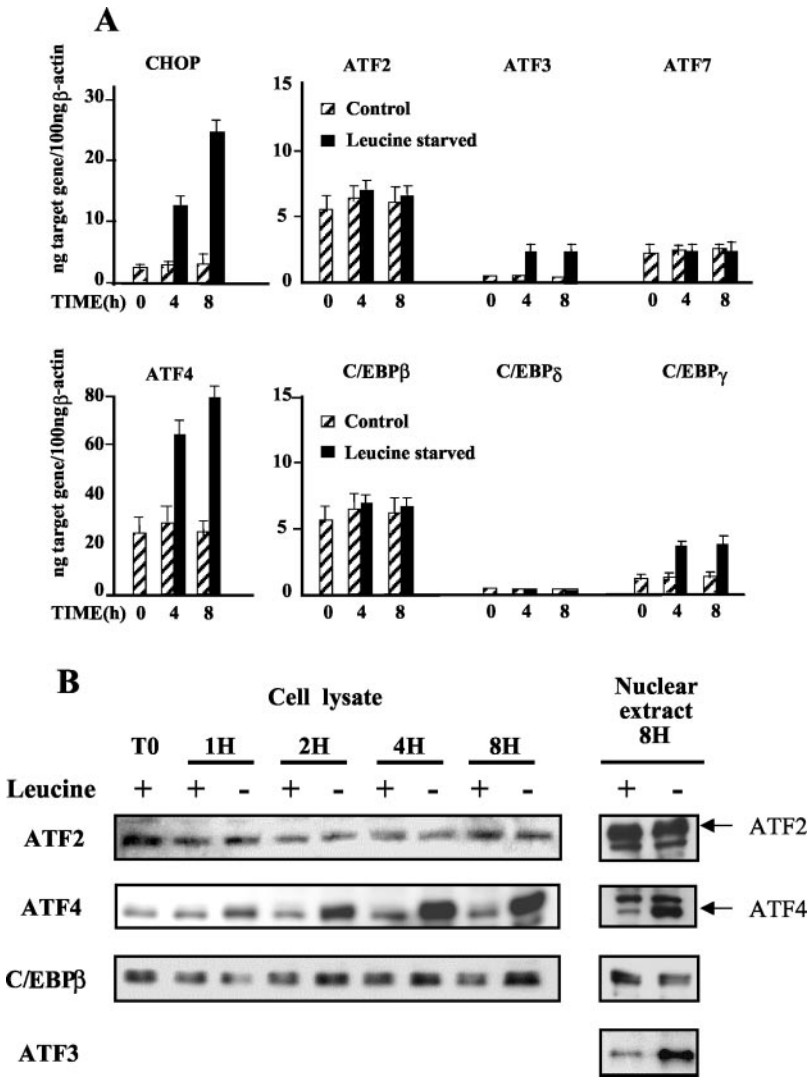

FIG. 2. **Effect of leucine starvation on the C/EBP and ATF transcription factors expression.** *A*, cells were incubated either in DMEM/F-12 or in DMEM/F-12 lacking leucine for 4 or 8 h. Total RNA were extracted, and real time RT-PCR was performed to quantify mRNAs as described under "Materials and Methods." *B*, immunoblot analysis of ATF2, ATF4, C/EBPβ, and ATF3 of extracts prepared from cells incubated in a leucine-deficient DMEM/F-12 media for the indicated period of time. Western blot analysis was performed either from whole cell lysate or from nuclear extracts.

scription factors that belong to the ATF family (ATF1, ATF3, ATF4, and ATF7) can potentially bind the AARE sequence together with members of the C/EBP family (C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ). To determine whether these transcription factors exhibit affinity for the *CHOP* AARE sequence, we performed EMSA in the presence of specific antibodies recognizing the ATF and C/EBP proteins (Fig. 1). A radiolabeled AARE oligonucleotide from −313 to −295 of the human *CHOP* proximal promoter was used as a probe. Experiments were performed using nuclear extracts from non-starved and 8-h-starved cells. As previously described (20), a major specific DNA-protein complex is detected after incubation of starved and non-starved HeLa nuclear extracts with ^{32}P-labeled *CHOP* AARE probe in the absence of specific antibody (*arrow* in Fig. 1). Fig. 1 (*A* and *B*) shows that antibodies against ATF2, ATF7, C/EBPβ, and C/EBPγ supershifted the *CHOP* AARE-bound complex with both starved and non-starved nuclear extracts, whereas antibodies against ATF3 and C/EBPδ supershifted the probe-protein complex only with the starved nuclear extracts. Because a faint supershifted complex was obtained with the anti-ATF4 antibody in leucine-starved condition, the experiment was repeated using, as a control, nuclear extracts from cells treated 5 h with a high dose of tunicamycin (5 μg/ml), which induces the ER stress response and leads to a high level of ATF4 expression (28). As shown Fig. 2*C*, an ATF4 supershift complex was obtained using nuclear extracts from both starved and tunicamycin-treated cells. Antibodies against ATF1 and C/EBPα did not supershift the AARE-protein complex (Fig. 1, *A* and *B*) while they produced a supershift under known control conditions (Fig. 1*D*).

Effect of Leucine Starvation on C/EBP and ATF Transcription Factors Expression—The marked discrepancy in the binding of transcription factors to the AARE sequence between nuclear extracts coming from starved and non-starved cells suggests that the cell content of these transcription factors is affected by leucine starvation.

A kinetic analysis showed that the *CHOP* mRNA content increases and reaches a plateau after 8 h leucine starvation (Fig. 2*A* and Ref. 19). Therefore, we measured the expression of the C/EBP and ATF transcription factors tested above in response to 4- and 8-h leucine starvation. Fig. 2*A* shows that ATF3, ATF4, and C/EBPγ mRNA content is increased in response to leucine starvation, whereas the expression level of other transcription factors is not affected. It is noticeable that the transcription factors ATF3, ATF7, and C/EBPδ are expressed at a very low level in HeLa cells.

Protein analysis shows (Fig. 2*B*) that the expression of ATF4 and ATF3 is markedly increased upon leucine starvation. The amount of ATF2 and C/EBPβ is not significantly affected by 8-h leucine deprivation. The expression levels of ATF7, C/EBPδ, and C/EBPγ are too low to be detected by Western blot, and ATF3 protein was measured only in nuclear extracts.

Role of the C/EBP and ATF Proteins in the Amino Acid Regulation of the AARE-dependent Transcription—We next de-

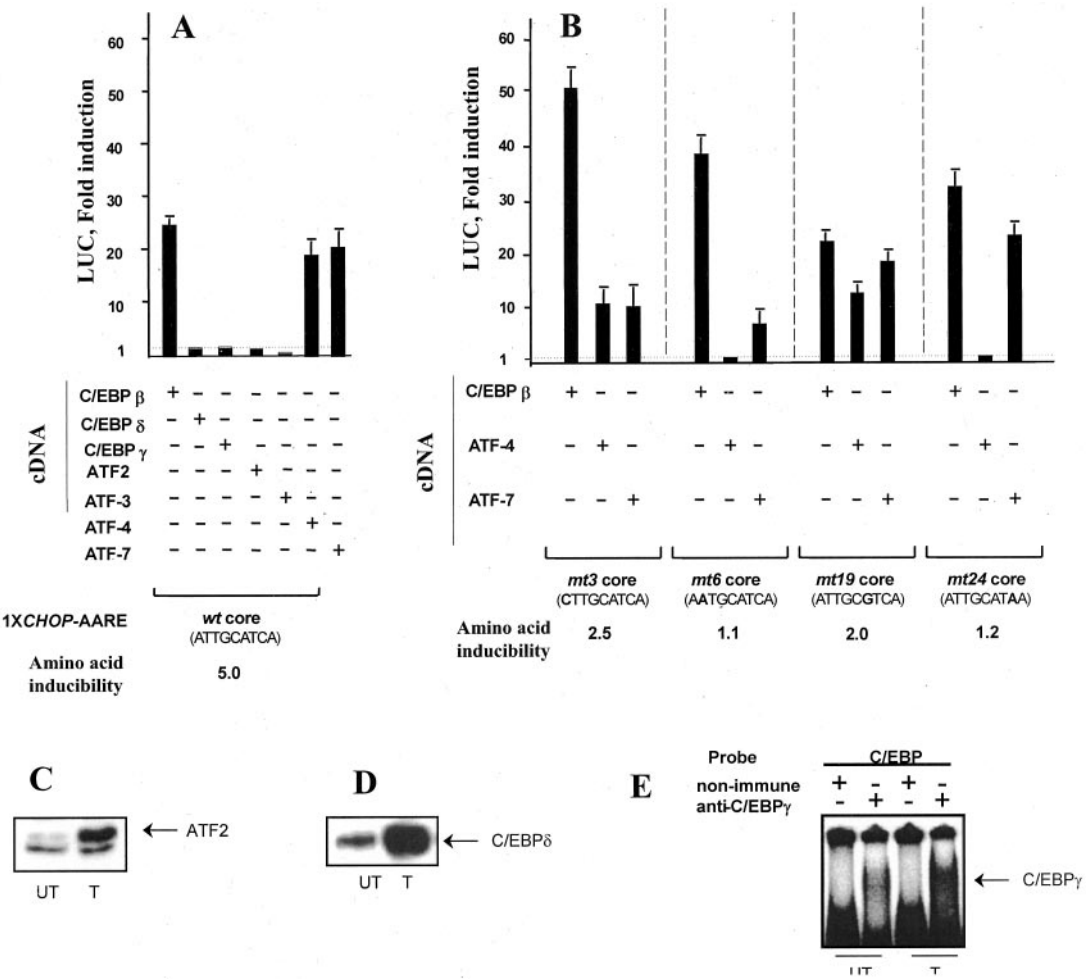

FIG. 3. **Effect of C/EBP and ATF proteins overexpression on the AARE-dependent transcription.** *A*, the construct (1X *CHOP* AARE LUC) containing a single copy of *CHOP* AARE (−313 to −295) inserted 5′ to the *TK* promoter driving LUC gene was used as reporter plasmid. Cells were transfected with a mixture containing the 1X *CHOP* AARE LUC (1 μg) and an expression plasmid (1 μg) encoding one transcription factor. For control experiments, cells were transfected with the reporter plasmid and the expression plasmid lacking the cDNA to be expressed. Two days after transfection cells were harvested for preparation of cell extracts and determination of LUC activity. Results are given as the "-fold induction" relative to the cells transfected with empty vector. To measure the amino acid inducibility, cells were transfected with the 1X *CHOP* AARE LUC construct. Twenty-four hours after transfection, cells were incubated for 16 h in complete DMEM (420 μM leucine) or in DMEM lacking leucine and harvested for preparation of cell extracts and determination of LUC activity. The amino acid inducibility is defined as the ratio of the relative LUC activity of leucine-starved cells to non-starved cells. For all the transfection experiments presented in this figure, a plasmid pCMV-βGAL was used as an internal control (see "Materials and Methods"). *B*, the same experiment was repeated using a reporter construct containing mutation on the *CHOP* AARE sequence. Mutations are indicated in *boldface*. *C* and *D*, Western blot showing that ATF2 and C/EBPδ are overexpressed following transfection of the expression plasmid (*UT* = untransfected; *T* = transfected). *E*, EMSA showing that C/EBPγ is overexpressed following transfection of the expression plasmid (*UT* = untransfected; *T* = transfected). We used a probe encoding the binding sequence for the C/EBP transcription factors family.

termined whether the transcription factors that bind the AARE sequence *in vitro* can regulate its transcriptional activity (Fig. 3). We cotransfected a LUC reporter driven by the AARE sequence with vectors that constitutively express transcription factors. Overexpression of C/EBPβ, ATF4, or ATF7 (Fig. 3*A*) stimulates the AARE-dependent transcription, whereas ATF3 expression decreases the basal AARE-dependent transcription about two times. Overexpression of ATF2, C/EBPδ, or C/EBPγ does not affect luciferase activity. The inability of these transcription factors to activate transcription is not due to a lack of expression, because transfection of plasmids encoding either ATF2, C/EBPδ, or C/EBPγ result in a strong increased of there expression as measured by Western blot or EMSA (Fig. 3, *C–E*). We have also checked that transfection of various amounts of these expression plasmid did not affect the AARE-dependent transcription (result not shown).

Among the transcription factors that bind the AARE sequence only C/EBPβ, ATF4, and ATF7 activate transcription. The next experiment was designed to assess their contribution to the regulation of the AARE-dependent transcription by amino acid starvation. In a previous study (29) we characterized the nucleotides, within the AARE sequence, essential for the transcriptional response to leucine starvation of the *CHOP* AARE. A series of point mutants of the AARE sequence were cloned upstream of the TK-LUC vector. Each construct was transiently transfected to measure its response to both leucine starvation and overexpression of one transcription factor. Fig. 3*B* shows that mt6 and mt24 mutants, which are not responsive to leucine starvation, are still activated by overexpression of C/EBPβ or ATF7. In contrast, these mutants are no more induced by ATF4 overexpression. It is worth noting that the mt3 and mt19 mutants, showing a reduced response to leucine starvation, exhibit also a reduced response to ATF4 overexpression. Taken together these results show that there is a good correlation, at the level of the AARE sequence, between the amino acid inducibility and the ability of ATF4 to activate the AARE-dependent transcription. Such a correlation does not exist with C/EBPβ or ATF7. These results suggest that ATF4

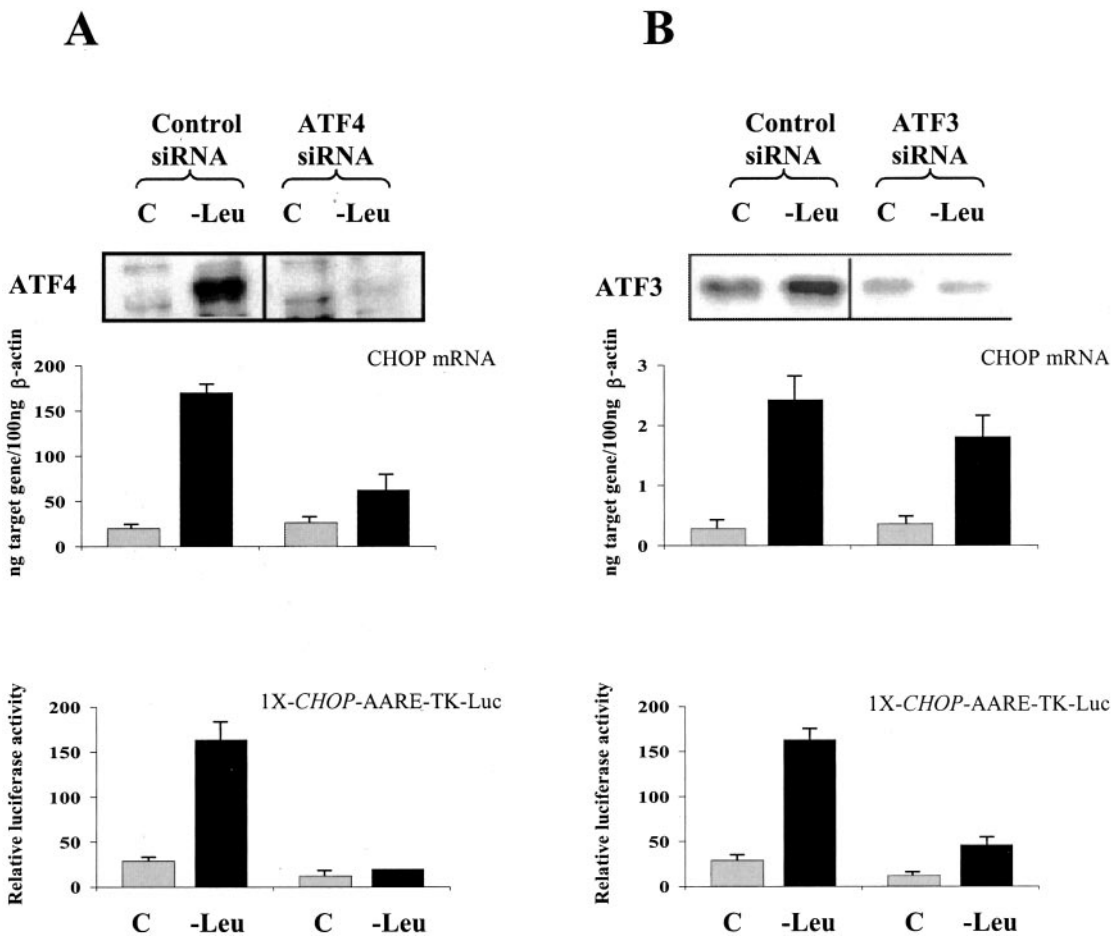

FIG. 4. **Effect of ATF4 or ATF3 knockdown on the amino acid regulation of CHOP expression.** Cells were transfected with ATF4 siRNA (*A*), ATF3 siRNA (*B*), or control siRNA (sequence mutated on 3 nucleotides). After 1 day, cells were transfected with the 1X *CHOP* AARE LUC reporter construct to measure the AARE-dependent transcription. Two days after siRNA transfection cell were incubated for 16 h in DMEM (*C*) or in DMEM lacking leucine (−*Leu*), and cells were then harvested to extract mRNA and proteins. ATF4 protein content was analyzed by Western blot, *CHOP* mRNA was quantified by real-time RT-PCR, and LUC activity was measured as described under "Materials and Methods."

plays an important role in the amino acid regulation of the AARE activity.

Effect of Specific Inhibition of ATF4 or ATF3 Expression on the Amino Acid Regulation of CHOP Expression—To assess the role of ATF4 in the amino acid regulation of *CHOP* expression, we measured the effect of leucine starvation on both *CHOP* mRNA content and AARE-dependent transcription in ATF4-deficient cells. We used small interfering double-stranded RNA (siRNA) transfection to specifically inhibit the expression of ATF4. Fig. 4*A* shows that ATF4-siRNA transfection dramatically decreases the ATF4 protein content and affects the response to leucine depletion: the induction of *CHOP* mRNA is dramatically reduced and the AARE-dependent transcription is abolished. In control cells (control siRNA-transfected cells) the response to leucine starvation is not affected. These data demonstrate that ATF4 expression is essential for the regulation of *CHOP* expression by leucine starvation.

Among the transcription factors able to bind the AARE sequence, ATF3 inhibits the AARE-dependent transcription when overexpressed. It has been suggested that ATF3 can bind the *CHOP* promoter in the region of the AARE sequence and inhibits its transcription (30–32). Moreover, It has recently been shown that ATF3 could also regulate another amino acid-regulated gene, *i.e.* asparagine synthetase (*AS*) (33). When overexpressed, it causes a concentration-dependent biphasic response on *AS* promoter-driven transcription. Taken together these data prompted us to investigate *CHOP* regulation by leucine starvation in ATF3-deficient cells. In the experiment presented in Fig. 4*B*, we measured the response to leucine starvation in cells transfected with ATF3-siRNA. The lack of ATF3 does not affect the induction of *CHOP* mRNA by leucine starvation. The basal level of the AARE-dependent transcription is decreased by the inhibition of ATF3 expression, but the induction of transcription by leucine starvation is not affected. Taken together these data suggest that ATF3 does not play an essential role in the leucine control of *CHOP* expression.

ATF4 Accumulation Subsequent to ER Stress Is Not Sufficient to Maximally Activate the AARE-dependent Transcription—The results presented above show that ATF4 plays an essential role in the amino acid control of gene expression. Because ATF4 is also involved in the ER stress pathway (28), we asked whether the AARE can mediate part of the ER stress response. In response to tunicamycin treatment or leucine deprivation, we measured *CHOP* expression, ATF4 protein content, and the transcriptional activity of both the *CHOP* promoter and the AARE. Both, a low concentration of tunicamycin (0.1 μg/ml) and leucine starvation lead to about the same increase of the *CHOP* transcript (Fig. 5*A*), the ATF4 protein (Fig. 5*B*), and the transcriptional activity of the *CHOP* promoter (Fig. 5*C*). Conversely, the AARE-dependent transcription (Fig. 5*D*) is induced only by leucine starvation (six times). Taken together these results show that ATF4 expression resulting from a treatment with a low concentration of tunicamycin is not sufficient to maximally activate the AARE-dependent transcription.

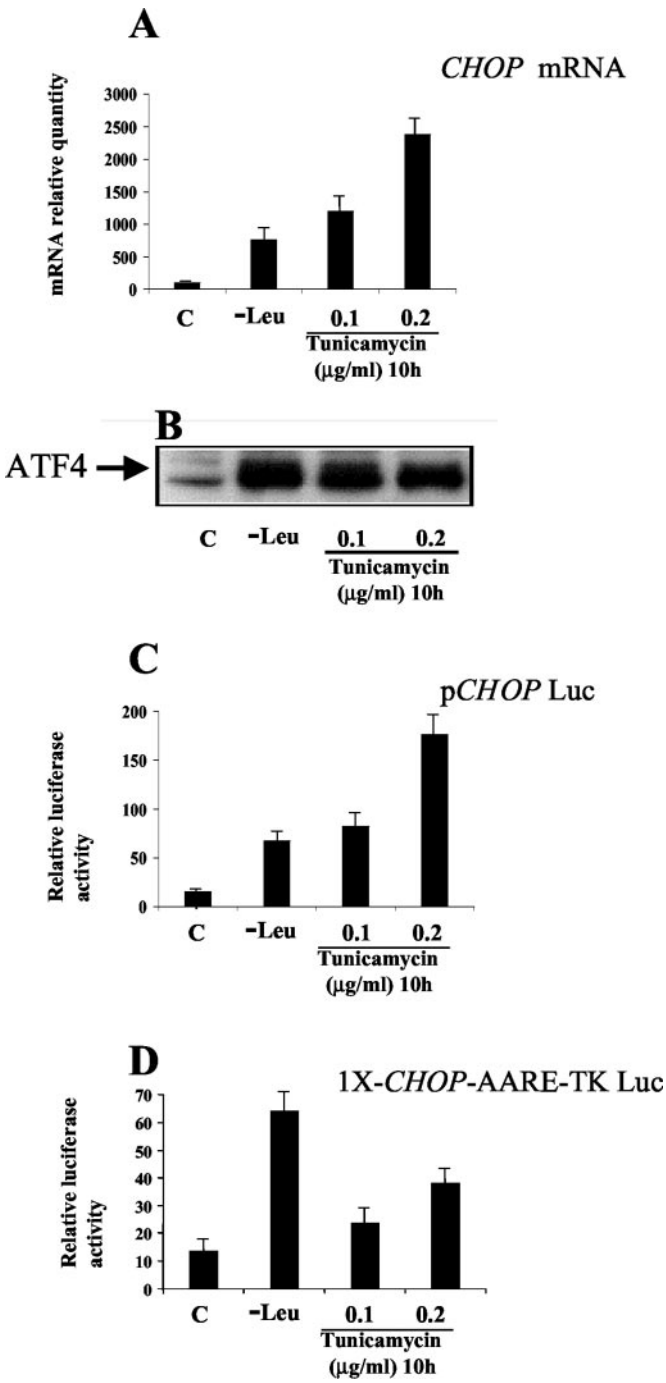

FIG. 5. **ATF4 accumulation subsequent to ER stress does not maximally activate the AARE-dependent transcription.** Cells were first transfected with reporter plasmids: p*CHOP* LUC to measure the transcriptional activity of the *CHOP* promoter (*C*) or 1X-*CHOP*-AARE LUC to measure the AARE-dependent transcription (*D*). Two days after transfection, cells were incubated for 10 h in DMEM or in DMEM lacking leucine or in DMEM containing 0.1 or 0.2 μg/ml tunicamycin. Cells were then harvested to measure *CHOP* mRNA (*A*), ATF4 protein content (*B*), and LUC activity (*C* and *D*) as described under "Materials and Methods."

ATF2 Phosphorylation Is Necessary for the Activation of the AARE-dependent Transcription—We have shown above that ATF4 induction alone is not sufficient to activate the AARE transcription. We had shown in a preceding article (20) that *CHOP* is no more induced by leucine starvation in ATF2-deficient cells. Therefore, we investigated whether another pathway, involving ATF2, is also necessary to increase the AARE-dependent transcription.

ATF2 is constitutively expressed, and, in response to various stimuli, its transcriptional activity as well as its histone acetyl transferase (HAT) activity are regulated by its phosphorylation on threonine 69 and 71 (34–38). Unphosphorylated ATF2 is transcriptionally silent, consequently, simple overexpression is not sufficient to activate transcription (Ref. 39 and Fig. 3*A*). To investigate whether ATF2 can activate the AARE in a functional assay, we used a constitutively active mutant of ATF2 (C2-ATF2) where the activation domain of ATF2 is exchanged with the constitutively active transcriptional activation domain of ATF4 (39). Steinmuller *et al.* (39) demonstrated that C2-ATF2 has the same properties that the wild type ATF2 has, in terms of specificity for both the binding site and the dimerization partner. Transient transfection experiments show that C2-ATF2 activates the AARE-dependent transcription (Fig. 6*A*, *lane 1*). To assess the contribution of ATF2 to the regulation of the AARE-dependent transcription by amino acid starvation, we have repeated the experiment described in Fig. 3 (*A* and *B*) using the C2-ATF2 construct. Fig. 6*A* shows that the mt6 and mt24 mutants, which are not responsive to leucine starvation, are not induced by C2-ATF2 overexpression. Finally, our results show that there is a good correlation, at the level of the AARE sequence, between the amino acid inducibility and the ability of C2-ATF2 to activate the AARE-dependent transcription.

Although the C2-ATF2 and wild type ATF2 have similar target sequences (39), the binding of ATF2 on the previously described mutants of the AARE has to be investigated. We performed a supershift assay (Fig. 6*B*) with anti-ATF2 antibody using oligonucleotides encoding the mutated form of the AARE sequence described in Figs. 3*B* and 6*A* as a probe. ATF2 binds the wild type as well as the mt3 and mt19 probes as shown by the presence of an ATF2-supershifted DNA-protein complex. In the case of the mt6 and mt24 probes, which are not responsive to leucine starvation, no ATF2-supershift was observed. These results demonstrate that the nucleotides within the *CHOP* AARE core sequence required to confer amino acid responsiveness are also essential for the binding of ATF2.

Because ATF2 is activated by phosphorylation and is required for the leucine regulation of *CHOP* expression, we investigated whether it is phosphorylated upon amino acid starvation (Fig. 6*C*). The phosphorylated form of ATF2 was estimated using a specific antibody recognizing the phosphothreonine 71. Leucine deprivation results in a rapid and sustained phosphorylation of ATF2 that appears after 1-h starvation and remains up to 8 h.

ATF4 and ATF2 Are Involved in the Regulation of Genes That Are Regulated by Amino Acids—Taken together our results demonstrate that leucine starvation activates two transcription factors that bind the AARE: it induces ATF4 expression and ATF2 phosphorylation. To determine whether the ATF2 and ATF4 dependence of the amino acid regulation of gene expression is restricted to *CHOP* or exists for other amino acid-regulated genes, we studied the expression of several genes regulated by amino acid availability in cells devoid of ATF2 or ATF4 expression. Among the known amino acid-regulated genes (40, 41), we studied genes expressed in both HeLa cells and mouse fibroblasts. As shown in Fig. 7 and in accordance with published results (40, 41), the mRNA content for these genes was up-regulated by amino acid starvation in control cells. However, the induction level can vary between cell lines: for example asparagine synthetase induction by leucine starvation is higher in HeLa cells than in mouse embryonic fibroblasts.

The lack of ATF4 results in a strong decrease or a loss of response to leucine starvation for all the genes tested. Analysis

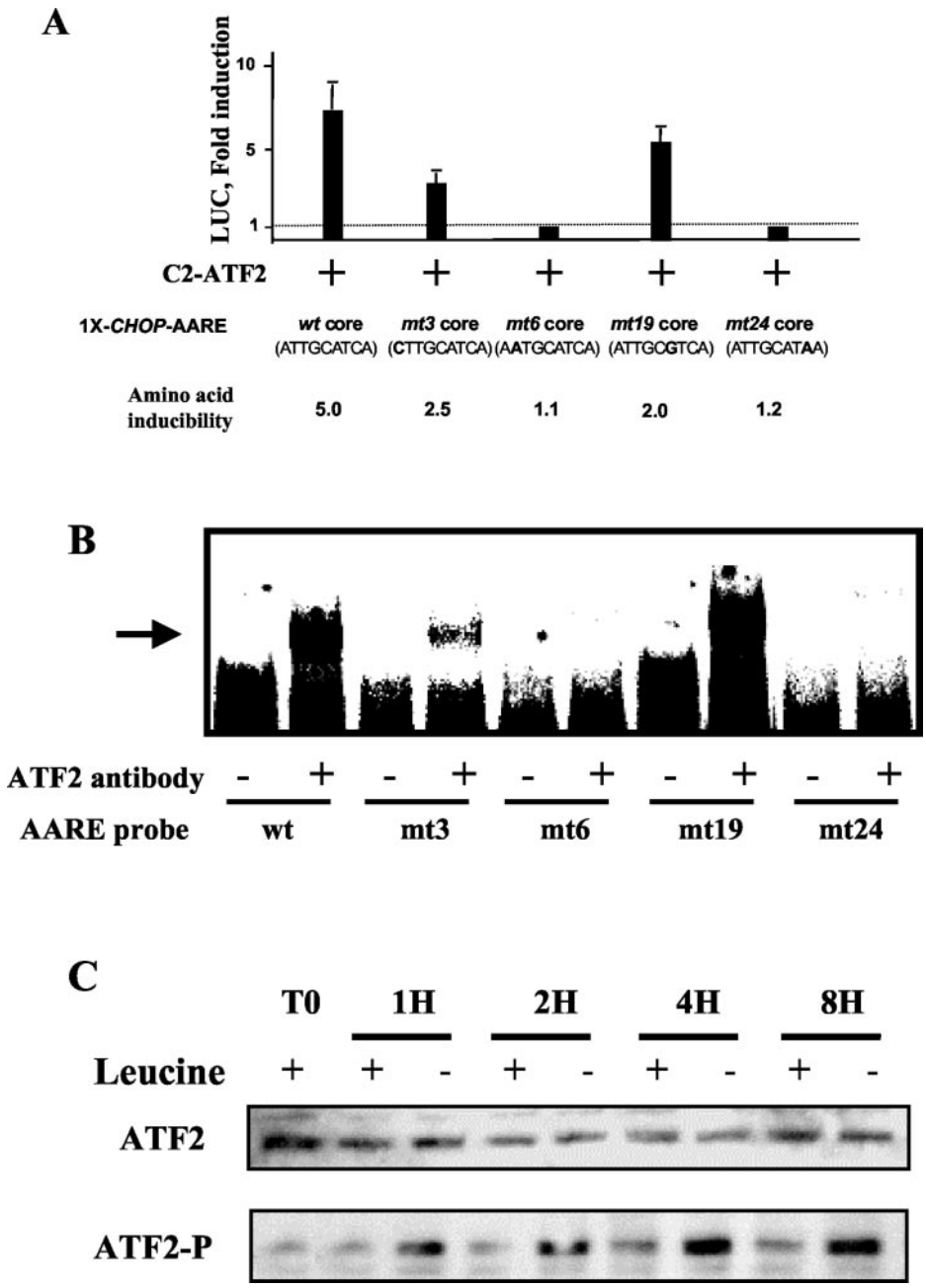

FIG. 6. **Role of ATF2 in amino acid regulation of CHOP expression.** *A*, the experiment was conducted as described Fig. 3 (*A* and *B*). HeLa cells were cotransfected with 1 μg of a reporter construct (1X *CHOP* AARE LUC) wild type or mutant and an expression plasmid encoding a constitutively active mutant of ATF2 (C2-ATF2). *B*, supershift assays using the anti-ATF2 antibody and nuclear extract from HeLa cells were performed as described under "Materials and Methods." Nuclear extracts were incubated with 1 μl of non-immune or anti-ATF2 serum and then incubated with the 19-bp wild-type (*wt*) or mutant (*mt3*, *mt6*, *mt19*, and *mt24*) *CHOP* AARE probes. Probes were diluted to obtain the same specific radioactivity. *C*, immunoblot analysis of ATF2 and phospho-ATF2 (Thr-71) in lysate of HeLa cells treated with a leucine-deficient medium for the indicated period of time.

of the ATF2−/− cells showed that leucine regulation is impaired for *CHOP*, ATF3, SARS, 4EBP1, and 4F2, whereas *AS* and YARS mRNA inducibility is strongly decreased but not totally abolished. Lack of ATF2 does not significantly affect the leucine regulation of SLC. These results show that (i) ATF4 has a critical role in the leucine regulation of all the genes tested and (ii) ATF2 plays an important role for the leucine control of most of these genes.

DISCUSSION

The *cis* DNA response element (AARE) located upstream from the transcription site (nucleotides −313 to −295) is essential for the amino acid regulation of the *CHOP* promoter. Sequence of the *CHOP* AARE shows some homology to the specific binding site of transcription factors that belong to the C/EBP and ATF families. However, we have documented (20) that several ATF or C/EBP binding sites, which are closely related to the AARE sequence, are not able to mediate amino acid inducibility suggesting that a precise set of transcription factors is required to mediate the amino acid response. We have previously demonstrated that the transcription factor ATF2 binds, *in vitro*, the *CHOP* AARE and is essential for the transcriptional activation of *CHOP* by leucine starvation. Our goals are 1) to characterize other transcription factors able to bind the AARE sequence and involved in the amino acid regulation of *CHOP* expression and 2) to understand the mechanisms involved in the activation of ATF2.

Here, we provide direct evidence that, ATF3, ATF4, ATF7, C/EBPα, C/EBPβ, C/EBPδ, and C/EBPγ have also the ability to bind the *CHOP* AARE sequence and could contribute to the regulation of transcription. However, our data demonstrate that among these transcription factors only ATF4 plays an essential role in the amino acid control of *CHOP* expression. Recently, D. Ron's group (28) have described a pathway for regulating gene expression in mammalian cells that is homologous to the well characterized yeast general control response to amino acid deprivation (42). Its components include an eIF2α kinases (GCN2) activated by amino acid starvation and

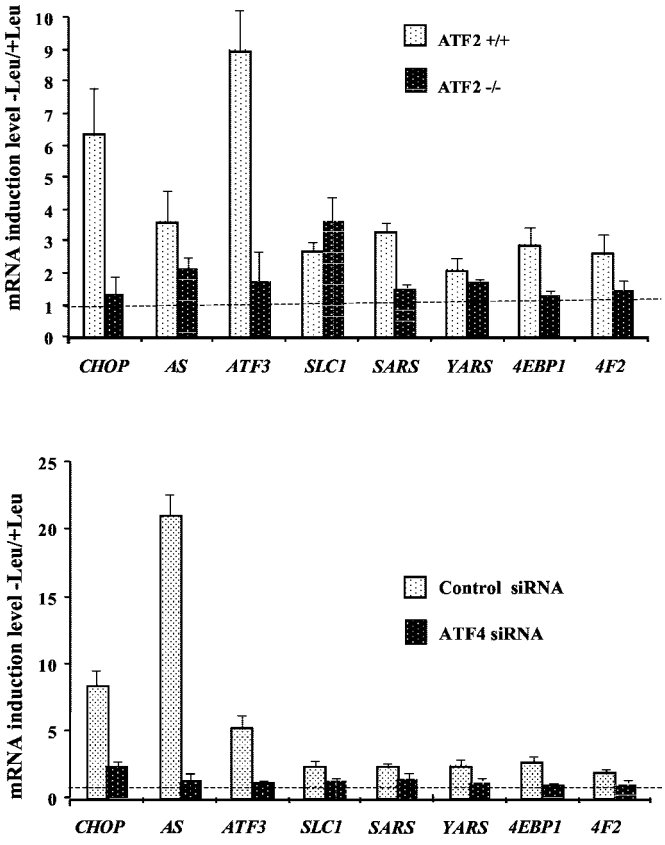

FIG. 7. **ATF4 and ATF2 are involved in the regulation of several amino acids regulated genes.** In the *upper panel* we used wild type and ATF2−/− mouse embryonic fibroblast cells; in the *lower panel* we used HeLa cells transfected with ATF4 siRNA or control siRNA. Cells were incubated either in DMEM medium or in leucine-deficient medium for 8 h. Total RNA were extracted, and real-time RT-PCR was performed to quantify mRNAs. Results are expressed as the mRNA induction level, defined as the ratio of the relative mRNA content of leucine-starved to non-starved cells. We measured the expression of the following genes: *CHOP* (C/EBP Homologous Protein), *AS* (Asparagine Synthetase), ATF3 (activating transcription factor 3), SLC1 (solute carrier family 1 (neutral amino acid transporter), member 5), SARS (seryl tRNA synthetase), YARS (tyrosyl-tRNA synthetase), 4EBP1 (eIF4E binding protein1), and 4F2 (4F2 antigen heavy chain).

ATF4 (homologous to the yeast transcription factor GCN4), whose translation is up-regulated when the general protein synthesis is decreased. Our present observations and the known characteristics of *CHOP* regulation by amino acid availability are consistent with the role of ATF4. First, synthesis of a regulatory protein is required prior to activation of *CHOP* transcription by leucine starvation (19). Consistent with this observation, ATF4 expression is enhanced by amino acid deprivation. Second, the binding of ATF4 on the AARE sequence is induced following leucine starvation. Third, ATF4 overexpression increases the AARE-dependent transcription. Fourth, mutations within the AARE sequence that impair the amino acid responsiveness also impair the transcriptional response to ATF4 overexpression. Finally, silencing of endogenous ATF4 gene abolished the transcriptional activation of *CHOP* by amino acid starvation. In addition, the ATF4 dependence of the amino acid regulation of gene expression is not restricted to *CHOP* but exists for other amino acid-regulated genes.

The role of ATF4 in the regulation of *CHOP* expression appears more complex than the role of GCN4 in yeast. Our experiments show that an increase in ATF4 expression following ER stress modestly activates the AARE-dependent transcription, whereas a similar ATF4 induction due to leucine starvation strongly induces the AARE activity. These data suggest that a second amino acid-dependent signal, other than ATF4 induction, occurs to maximally activate the AARE-dependent transcription. This signal could modulate the transcriptional activity of ATF4 through (i) its interactions with another partner, (ii) its interactions with chromatin components, (iii) its post-translational modification (for example phosphorylation) (43), (iv) its nuclear localization (44). However, it is worth noting that enforced overexpression of ATF4 partially overcomes the need for this amino acid-dependent signal. Indeed, transient transfection of ATF4, which results in a large overexpression of ATF4, maximally activates the AARE activity.

The data presented here, in addition with previously published results (20), demonstrate that ATF2 has an essential role in the transcriptional activation of *CHOP* by leucine starvation. ATF2 binds its target promoter sequence as a homodimer or as a heterodimer with a restricted group of other bZip proteins. The transacting capacity of ATF2 depends on its phosphorylation on N-terminal residues (Thr-69 and Thr-71) and on its partner (34–38). Here, we show that ATF2 is phosphorylated on Thr-71 upon leucine starvation. According to the literature ATF2 is phosphorylated on this residue by the mitogen-activated protein kinase (MAPK) members ERK, p38 and JNK/stress-activated protein kinase (37) in response to serum or interleukin treatment or various cellular stress. It has been shown that ERK and p38 MAPK are not activated by starvation in a single amino acid (45). However, the c-Jun N-terminal kinase 1 (JNK1) is slightly induced by leucine starvation (45) with a kinetic of activation that cannot explain the rapid and sustained phosphorylation of ATF2. Therefore, the kinase involved in the phosphorylation of ATF2 in response to leucine starvation remains to be identified.

ATF2 dependence of the amino acid regulation of gene expression is variable according to the tested gene. Although ATF2 knockout totally abolishes the leucine control of most of the tested genes, the amino acid control of certain genes remains partially or totally insensitive to ATF2 expression. ATF2 has an intrinsic histone acetyl transferase (HAT) activity that is also regulated by phosphorylation on threonines 69 and 71 (38). It can be hypothesized that the HAT activity could modulate the interactions between ATF2 and other partners involved in the control of transcription (transcription factors, chromatin components, and others) and thus modulates the amino acid regulation of gene expression.

In addition to ATF2 and ATF4, other transcription factors that belong to the ATF or C/EBP families have the ability to bind *in vitro* the AARE sequence. ATF3, ATF7, and C/EBPβ bind the AARE sequence and affect the transcriptional regulation. However, it is not likely that they play a major function in the amino acid control of the endogenous *CHOP* transcription. Indeed, ATF7 and C/EBPβ are both able to activate transcription of mutated sequences of the AARE that are not responsive to leucine starvation. Moreover, C/EBPβ knockout does not impair the amino acid regulation of *CHOP* expression (29), and the lack of ATF3 does not affect *CHOP* induction by leucine starvation. However, we cannot exclude that these transcription factors could belong to the protein complex, which binds the AARE sequence and could indirectly modulate the response to amino acid starvation.

It is now clear that many transcription factors that belong to the C/EBP or ATF families are able to bind the AARE sequence. A combination of several transcription factors and regulatory proteins could bind the AARE sequence to precisely control the rate of transcription. Our data clearly show that ATF2 and ATF4 are involved in the amino acid regulation of *CHOP* tran-

scription. Although these two proteins can coimmunoprecipitate (46), we have no evidence that ATF2 and ATF4 may form a dimer that binds the AARE sequence, but they could be included in a larger regulatory protein complex. For example, it has been shown that ATF2 interacts with at least two transcriptions factors (CP1 and NF1) in a protein complex that regulates transcription of the fibronectin gene (47). We have also shown that, beside the GCN2 pathway that leads to ATF4 induction, other amino acid-dependent signals are required to phosphorylate ATF2 and to maximally activate the AARE-dependent transcription. The nature of these signals and their links with the GCN2 pathway remain to be investigated. 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 metabolic control in mammalian cells.

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