Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels

Alain Bruhat, Céline Jousse, Xiao-Zhong Wang, David Ron, Marc M. Ferrara, Pierre Fafournoux

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 28, Issue of July 11, pp. 17588–17593, 1997

Printed in U.S.A.

Amino Acid Limitation Induces Expression of *CHOP*, a CCAAT/Enhancer Binding Protein-related Gene, at Both Transcriptional and Post-transcriptional Levels*

(Received for publication, February 11, 1997, and in revised form, March 31, 1997)

Alain Bruhat‡§, Céline Jousse‡¶, Xiao-Zhong Wang‖, David Ron‖, Marc Ferrara‡, and Pierre Fafournoux‡ ‡‡**

From the ‡Unité de Nutrition Cellulaire et Moléculaire, INRA de Theix, 63122 Saint Genès Champanelle, France and ‖Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, New York 10016

In mammals, plasma concentrations of amino acids are affected by nutritional or pathological conditions. Here we examined the role of amino acid limitation in regulating the expression of *CHOP*, a CCAAT/enhancer binding protein (C/EBP)-related gene. CHOP protein is capable of interacting with other C/EBPs to modify their DNA binding activities and may function as a negative regulator of these transcription factors. Our data show that leucine limitation in human cell lines leads to induction of *CHOP* mRNA and protein in a dose-dependent manner. *CHOP* mRNA induction is rapidly reversed by leucine replenishment. Elevated mRNA levels result from both an increase in the rate of *CHOP* transcription and an increase in the *CHOP* mRNA stability. Using a transient expression assay, we show that a promoter fragment, when linked to a reporter gene, is sufficient to mediate the regulation of *CHOP* expression by leucine starvation in HeLa cells. In addition, we found that decreasing amino acid concentration by itself can induce *CHOP* expression independently of a cellular stress due to protein synthesis inhibition. Moreover, *CHOP* expression is induced at leucine concentrations in the range of those observed in blood of protein-restricted animals suggesting that amino acids can participate, in concert with hormones, in the regulation of gene expression.

Cells regulate gene expression in response to changes in the external environment. Metabolite control of gene expression has been well documented in prokaryotes and lower eukaryotes. Specific mechanisms have evolved to allow these organisms to quickly metabolize various molecules based on their availability in the external medium (1, 2).

However, much less is known about the response of multicellular organisms to nutrient variations. The control of gene expression differs in many aspects from those operating in single cell organisms and involves complex interactions of hormonal, neuronal, and nutritional factors. It has been shown that major (carbohydrates, fatty acids, sterols) or minor (minerals, vitamins) dietary constituents participate, in concert with many hormones, in the regulation of gene expression in response to nutritional changes (3–7). There is considerably less information available concerning the control of mammalian gene expression by amino acids. However, it has been shown that starvation of one essential amino acid causes a specific increase in mRNA abundance of certain genes including c-*myc*, c-*jun*, ornithine decarboxylase (8), asparagine synthetase (9), the mammalian equivalent of ribosomal protein L-17 (10), the insulin-like growth factor binding protein gene (11). Moreover, Marten *et al.* (12) have shown that the abundance of several different mRNAs is affected by amino acid starvation. In this study the greatest induction in response to amino acids starvation was exhibited by the *CHOP* gene. However, little is known about the molecular mechanisms involved in gene regulation by amino acids. It has only been shown that the induction of asparagine synthetase gene by amino acid starvation involves both transcriptional and post-transcriptional mechanisms (9). These authors have characterized *cis*-acting elements involved in transcriptional regulation of that gene in response to amino acid starvation.

CHOP (also called *gadd153*) is a mammalian gene whose expression is also induced in all tested cells by a wide variety of stresses and agents (13–16). *CHOP* encodes a small nuclear protein related to the CCAAT/enhancer-binding protein (C/EBP)[1] family of transcription factors. Members of the C/EBP family have been implicated in the regulation of processes relevant to energy metabolism (17), cellular proliferation, differentiation, and expression of cell type-specific genes (18–20). By forming heterodimers with the members of the C/EBP family, CHOP protein can influence gene expression as both a dominant negative regulator of C/EBP binding to one class of DNA targets and by directing CHOP-C/EBP heterodimers to other sequences (21–26).

In mammals, plasma concentrations of glucose and free amino acids are markedly affected by nutritional or pathological conditions (27, 28). Carlson *et al.* (15) have shown that *CHOP* mRNA expression is induced by glucose deprivation in mammalian cell lines, suggesting a close relationship between nutrient variation and *CHOP* expression. In the present study we have examined the role of amino acids in the regulation of *CHOP* expression. We demonstrate that amino acid limitation, in conditions which do not inhibit protein synthesis, can induce

* This work was supported in part by grants from the Institut National de la Recherche Agronomique and the Fondation pour la Recherche Médicale. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a fellowship from the Société de Secours des Amis des Sciences.

¶ Recipient of a French Ministère de l'Eduction Nationale et de l'Enscignment Supirieur (MENESR) pre-doctoral scholarship.

** Supported by National Institutes of Health Grants DK47119 and ES08681 and is a Leukemia Society of America Stephen Birnbaum Scholar.

‡‡ To whom correspondence should be addressed. Tel.: 33 4 73 62 45 62; Fax: 33 4 73 62 45 70; E-mail: fpierre@clermont.inra.fr.

[1] The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum Eagle's medium; CHOP, C/EBP homologous protein.

CHOP expression. Particularly, we show that leucine starvation induces *CHOP* expression through both transcriptional and post-transcriptional mechanisms. The implication of these findings are discussed in a general context of the control of mammalian gene expression by amino acids in various nutritional conditions.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions—Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium F12 (DMEM/F12) (Sigma) containing 10% (HeLa and HepG2) or 20% (Caco-2) fetal bovine serum. When indicated, DMEM/F12 lacking leucine was used. For other amino acid or glucose starvation experiments, MEM medium (Life Technologies, Inc.) was used. For amino acid starvation experiments 10% dialyzed calf serum was used.

RNA Isolation and Northern Blot Analysis—Total RNA was prepared as described previously (29). Northern blots were performed according to the procedure of Sambrook *et al.* (30). The membranes were UV cross-linked and then prehybridization was carried out for 2 h at 55 °C in 50% formamide, 6 × SSC, 5 × Denhardt's reagent, 0.5% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 10 μg/ml yeast tRNA. The human *CHOP* cDNA (BH1), generously provided by Dr. N. J. Holbrook (31), was used as a probe. BH1 plasmid was linearized by *Pst*I, and ^{32}P-riboprobes were synthesized (30) using T7 RNA polymerase (Promega). Hybridization was carried out for 16 h at 55 °C. The membranes were washed for 15 min at 55 °C successively in 2 × SSC containing 0.1% SDS, 0.5 × SSC containing 0.1% SDS, 0.1 × SSC containing 0.1% SDS. Labeled bands were detected by autoradiography. Autoradiogram signals were quantified by using a densitometric scanner (Appligene) and NIH image software. To control for variation in either the amount of RNA in different samples or loading errors, all blots were rehybridized with an oligonucleotide probe corresponding to 18 S RNA. All densitometric values for *CHOP* mRNA were normalized to 18 S RNA values obtained on the same blot. Relative *CHOP* mRNA was determined as the ratio of *CHOP* mRNA and 18 S RNA.

DNA Transfection and CAT Assay—HeLa cells (5×10^5) were plated in 60-mm diameter dishes and transfected by the calcium phosphate coprecipitation method as described previously (32). Ten micrograms of CAT plasmid were transfected into the cells along with 2 μg of pCMV-βGal, a plasmid carrying the bacterial β-galactosidase gene fused to the human cytomegalovirus immediate-early enhancer/promoter region, as an internal control. Cells were exposed to the precipitate for 16 h, washed twice in phosphate-buffered saline, and then incubated with DMEM/F12 containing 10% fetal calf serum. Twenty-four hours after transfection, cells were amino acid-starved for the desired time and then collected for CAT assay (33). The protein concentration of the cell extracts was determined using the BCA method (34). β-Galactosidase activity was measured as described by Hall *et al.* (35) and used to calibrate transfection efficiency. Relative CAT activity was given as a percentage of pSV2CAT activity. All values are the means calculated from the results of at least three independent experiments.

Primer Extension—Total cellular RNA from transfected cells was isolated as described above. A 20-base pair oligonucleotide (5′-CAACGGTGGTATATCCAGTG-3′), complementary to the DNA sequence located 11–30 base pairs downstream from the translation initiation site of the *cat* gene, was end-labeled with T_4 polynucleotide kinase (Eurogentec) and then used for primer extension as described previously (36).

Nuclear Run-on Transcription Assays—*In vitro* transcription experiments in isolated HeLa cell nuclei were carried out essentially as described by Liu *et al.* (37). RNA was labeled with [^{32}P]UTP and then hybridized to filter-bound cDNAs of *CHOP* (31), ribosomal S26 protein (38), and pBluescript DNA (Stratagene). Hybridization with labeled RNA was performed at 45 °C for 24 h. The filters were washed twice for 15 min in 5 × SSC plus 0.2% SDS at 45 °C, followed by three washes in 2 × SSC plus 0.2% SDS at 45 °C. Radioactive dots were visualized and quantified by using a PhosphorImager (Bio-Rad) and the MOLECULAR ANALYST software.

Protein Synthesis Measurements—HeLa cells were incubated for 16 h in DMEM/F12 containing 420, 140, 70, 35, or 0 μM leucine. During the last 3 h of incubation, 0.5 μCi/ml [^{35}S]methionine were added. The medium was then removed, and the cells were incubated for 30 min in cold 5% trichloroacetic acid. The wells were washed once with trichloroacetic acid and three times with water. The radioactivity incorporation into trichloroacetic acid-precipitable material was measured by liquid scintillation counting after protein solubilization in 0.1 M NaOH plus 0.5% SDS. Results are given as a percentage of methionine incorporation in cells incubated in DMEM/F12 control medium.

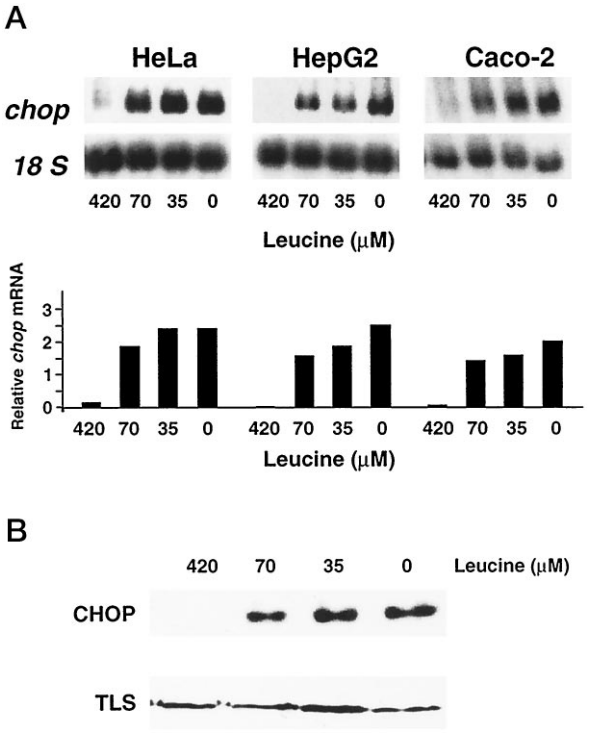

FIG. 1. **Effect of leucine limitation on *CHOP* mRNA and protein expression.** *A*, subconfluent HeLa, HepG2, and Caco-2 cells were incubated for 16 h in DMEM/F12 containing the indicated leucine concentrations. 420 μM leucine correspond to DMEM/F12 control medium. Total RNA was extracted, and Northern blots were prepared as described under "Materials and Methods." The blots were hybridized with a labeled probe corresponding to *CHOP*. The *CHOP* mRNA migrates as a single 0.9-kilobase pair transcript. The same membranes were rehybridized with an 18 S probe to normalize for RNA loading. The quantification of these data is shown below the signal for *CHOP* and 18 S on RNA blots. *B*, subconfluent HeLa cells were incubated for 16 h in DMEM/F12 containing the indicated leucine concentrations. Whole cell lysates were prepared and probed for the presence of CHOP by Western blot analysis as described under "Materials and Methods." The blot was then probed with an anti-TLS antibody as an internal control.

Western Blot Analysis—Cells were lysed in a SDS-containing buffer (0.1 M Tris-HCl, pH 6.8, 1% SDS) and immediately boiled for 5 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. CHOP and the ubiquitous nuclear protein TLS were detected in D. Ron's laboratory as previously described (39, 40).

RESULTS

Induction of CHOP mRNA Expression by Leucine Limitation—To understand the regulation of gene expression by amino acids at a molecular level, we have studied the regulation of *CHOP* expression in response to leucine limitation because (i) leucine is an essential amino acid that is poorly utilized by cells during a 16-h incubation period (data not shown), (ii) leucine, which is transported by system L, is rapidly equilibrated through the cell membrane (41, 42), and (iii) Marten *et al.* (12) have shown that leucine depletion strongly induces *CHOP* expression. To test the possibility that leucine concentration can influence *CHOP* expression, HeLa, HepG2, or Caco-2 cells were incubated for 16 h in medium containing different concentrations of leucine. As shown in Fig. 1*A*, *CHOP* mRNA levels were very low in each cell type in control medium containing 420 μM leucine and were inversely proportional to the leucine concentration in the medium, ranging from 15- to 30-fold over the control value. These results indicate that the expression of *CHOP* mRNA in human cells is regulated in response to changes in leucine concentration. Fig. 1*B* shows

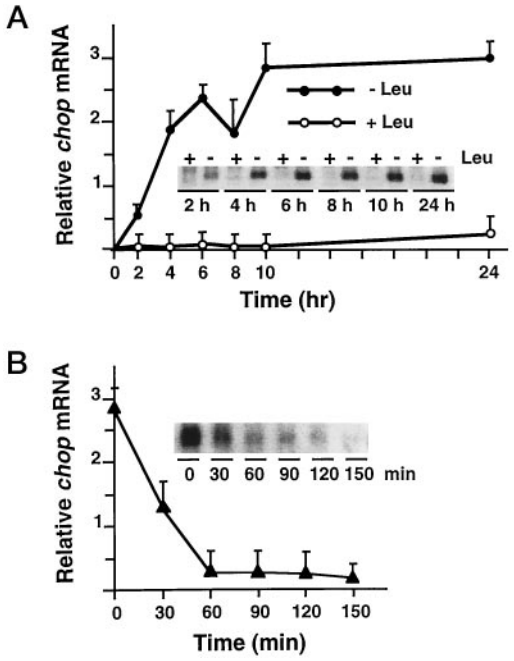

FIG. 2. **Induction and reversal of *CHOP* expression by leucine starvation.** *A*, HeLa cells were incubated either in DMEM/F12 (+*Leu*) or in DMEM/F12 lacking leucine (−*Leu*) and harvested for RNA isolation after the indicated incubation times. Northern blot analysis was performed as described under "Materials and Methods." The blots were hybridized with a *CHOP* probe and rehybridized with an 18 S probe to normalize for RNA loading. *B*, following 16 h of leucine starvation, 420 μM leucine was added to the culture medium of HeLa cells, and the RNA was harvested at the times indicated. The *error bars* represent standard deviation from the mean of two independent experiments in duplicate.

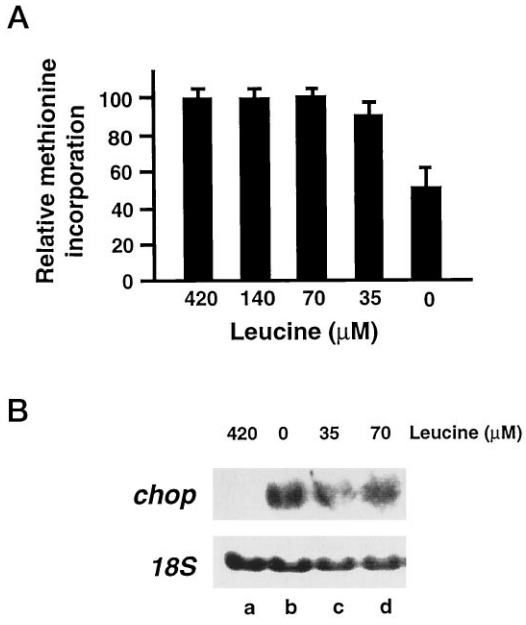

FIG. 3. **Effect of leucine concentration on protein synthesis and *CHOP* mRNA accumulation.** HeLa cells were incubated for 16 h in DMEM/F12 containing the indicated leucine concentrations. 420 μM leucine correspond to DMEM/F12 control medium. *A*, the protein synthesis was measured by [^{35}S]methionine incorporation during the last 3 h of incubation as described under "Materials and Methods." *B*, the cells were incubated for 16 h with the indicated leucine concentration. Northern blot analysis was performed as described under "Materials and Methods." The blots were hybridized with a *CHOP* probe and rehybridized with an 18 S probe to normalize for RNA loading.

that the increase in *CHOP* mRNA levels results in the increase in the CHOP protein. Kinetic analysis of *CHOP* mRNA level in HeLa cells exposed to medium lacking leucine indicated that mRNA was detectable 2 h after starvation, and a maximum level was reached after 10–12 h (Fig. 2*A*). To determine whether the induction of *CHOP* expression by leucine starvation is reversible by leucine replenishment, 420 μM leucine was added to the culture medium of HeLa cells incubated for 16 h in leucine-free medium. Fig. 2*B* clearly shows that leucine addition resulted in a rapid loss of *CHOP* mRNA expression with levels declining over 90% by 1 h following the addition of leucine.

Inhibition of Protein Synthesis Is Not Responsible for Induction of CHOP mRNA Expression—To determine whether leucine limitation affects protein synthesis, HeLa cells were incubated in medium containing different concentrations of leucine and then [^{35}S]methionine incorporation in the acid-precipitable fraction was measured (Fig. 3*A*). Cells incubated in medium lacking leucine showed a 40% reduction of methionine incorporation into total protein together with a drastic increase in *CHOP* mRNA level (Fig. 3*B*, *lane b*). However, cells incubated in medium containing 35 or 70 μM leucine gave no significant reduction of the global protein synthesis, whereas *CHOP* mRNA expression was significantly increased (Fig. 3*B*, *lanes c* and *d*). These observations are consistent with the idea that inhibition of protein synthesis is not responsible for the induction of *CHOP* mRNA expression.

The Induction of CHOP Expression by Leucine Starvation Involves Both Transcriptional and Post-transcriptional Mechanisms—Leucine starvation could increase *CHOP* mRNA expression either by increasing the rate of transcription or by stabilizing existing transcripts, or through both mechanisms. Nuclear run-on experiments provided evidence that the rate of *CHOP* transcription was increased by leucine starvation (Fig. 4*A*). Four hours of leucine starvation increased dramatically the transcription of *CHOP* (21-fold), while the transcription of the *S26* ribosomal gene remained unchanged. To determine whether leucine starvation can affect the half-life of *CHOP* mRNA, HeLa cells were first incubated for 16 h in medium lacking leucine and then incubated with actinomycin D (4 μg/ml) in the presence or absence of 420 μM leucine, and total mRNA was extracted from cells at various times. As shown in Fig. 4*B*, addition of leucine resulted in a rapid decline in *CHOP* mRNA levels. In starved cells, the *CHOP* mRNA half-life was increased about 3-fold compared with cells incubated in the control medium. These findings indicate that leucine starvation elevates *CHOP* mRNA levels both by increasing the rate of *CHOP* transcription and by enhancing the stability of *CHOP* mRNA. To assess the importance of protein synthesis for the increase of *CHOP* mRNA expression during leucine starvation, cells were leucine-starved and treated with cycloheximide for 4 h. As shown in Fig. 4*C*, cycloheximide present during leucine starvation prevented the accumulation of *CHOP* mRNA. This result indicates that the increase in *CHOP* mRNA during leucine starvation is dependent on *de novo* protein synthesis.

Regulation of CHOP Promoter Activity by Leucine Starvation—To analyze the role of *CHOP* promoter in transcription activation by leucine starvation, a chimeric gene (p*CHOP*-CAT) containing the 5′-flanking sequence from nucleotides −954 to +91 fused to the *cat* gene (31) was transiently transfected in HeLa cells. The data presented in Fig. 5*A* (summarized in the graph of Fig. 5*B*) show that CAT activity expressed under the control of the *CHOP* promoter was induced 7-fold by 16 h of leucine starvation, whereas CAT activity expressed from the pSV2CAT construct used as a control was not induced. These results gave direct evidence that regulation of *CHOP* transcription by leucine starvation is mediated through the promoter sequence situated between nucleotide position −954 and +91. Similar increased levels of CAT activity were also observed

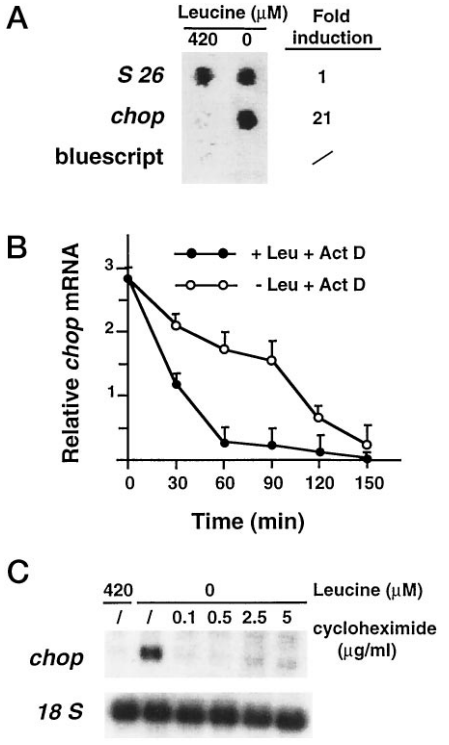

FIG. 4. **Transcriptional and post-transcriptional regulation of *CHOP* by leucine starvation.** *A*, nuclear run-on analysis of *CHOP* transcription. HeLa cells were incubated for 4 h in DMEM/F12 control medium (420 μM) or in DMEM/F12 lacking leucine (0 μM). ^{32}P-Labeled RNA isolated from HeLa cells was hybridized to filter-bound DNAs of ribosomal *S26*, *CHOP*, and bluescript vector. The fold induction was determined as the ratio of mRNA expressed in leucine-starved to non-starved media. The numbers are the average of two separate experiments. *B*, effect of leucine starvation on *CHOP* mRNA stability. HeLa cells were initially incubated for 16 h in DMEM/F12 lacking leucine. At this point (time 0), cells were incubated in the presence of 4 μg/ml actinomycin D (*Act D*), either in DMEM/F12 (+*Leu* + *Act D*) or in DMEM/F12 lacking leucine (−*Leu* + *Act D*). Total RNA was extracted from each group of cells after the indicated incubation times. Northern blot analysis was performed as described under "Materials and Methods." Blots were hybridized with a *CHOP* probe and rehybridized with an 18 S probe to normalize for RNA loading. The *error bars* represent standard deviation from the mean of two independent experiments in duplicate. *C*, effect of cycloheximide on *CHOP* mRNA accumulation. HeLa cells were incubated for 4 h in DMEM/F12 (420 μM) or in DMEM/F12 lacking leucine (0 μM) with 0.1, 0.5, 2.5, or 5 μg/ml cycloheximide as indicated. Northern blot analysis was performed as described under "Materials and Methods."

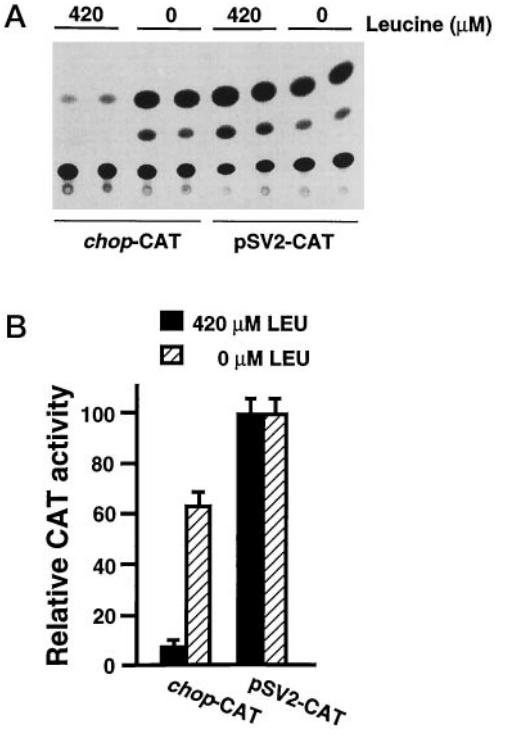

FIG. 5. **Regulation of CAT activity under the control of the *CHOP* promoter in leucine-starved HeLa cells.** The plasmid p*CHOP*-CAT corresponds to the human *CHOP* promoter region from nucleotide −954 to +91 fused to the bacterial chloramphenicol acetyltransferase (*CAT*) gene (31). HeLa cells were transiently transfected with plasmid p*CHOP*-CAT or with plasmid pSV2CAT along with plasmid pCMV-βGal carrying the β-galactosidase gene as described under "Materials and Methods"; 24 h after transfection, cells were incubated for 16 h in DMEM/F12 (420 μM) or in DMEM/F12 lacking leucine (0 μM) and harvested for preparation of cells extracts and CAT activity determination. *A*, autoradiogram corresponding to CAT assays from p*CHOP*-CAT and pSV2CAT. *B*, relative CAT activity of these constructs normalized with respect to the plasmid pCMV-βGal as described under "Materials and Methods."

with transfection of p*CHOP*-CAT into HepG2 and Caco-2 cells (data not shown). To correlate CAT activity and amounts of CAT mRNA transcribed under leucine-starved and non-starved conditions, primer extension experiments were performed. As shown in Fig. 6, under leucine starvation, the amounts of CAT mRNA initiating at the correct start site of the promoter were much higher (*lane b*) than those transcribed in normal conditions (*lane a*), and the levels of CAT mRNA derived from pSV2CAT remained unchanged (*lanes c* and *d*). These results show that the degree of induction of p*CHOP*-CAT mRNA expression (6–7-fold) is in agreement with the degree of induction determined in CAT assays and indicate that, under our experimental conditions, leucine starvation does not affect significantly translation of the CAT mRNA.

To determine whether the *CHOP* promoter-driven CAT induction is consistent with that described for the endogenous *CHOP* mRNA, we examined the characteristics of the *CHOP* promoter activity in response to leucine limitation. Fig. 7*A* shows that the transcriptional activity from *CHOP* promoter was enhanced by a decrease in leucine concentration in a dose-dependent manner. Furthermore, kinetic analysis of the *cat* gene expression revealed that maximal CAT activity induction was reached 16 h after starvation (Fig. 7*B*).

Specificity of the CHOP Promoter Response to Amino Acid Starvation—Starvation of other amino acids was tested for their abilities to influence *CHOP* promoter-driven CAT expression in HeLa cells (Fig. 8). The most potent amino acids increasing CAT activity level appeared to be methionine, lysine, arginine, phenylalanine, and threonine. They produced about the same induction level of CAT activity as that obtained with leucine (5–8-fold). Glutamine, aspartate, asparagine, cysteine, proline, and glutamate had minor but consistent increasing effects on CAT activity (2- to 3-fold). In contrast, alanine and serine had no significant effect on the level of CAT activity. These results provide evidence that the degree of effectiveness for each amino acid on the *CHOP* promoter activity varied widely. Moreover, this part of *CHOP* promoter also responds to glucose deprivation. In these experimental conditions, the induction in CAT activity due to glucose deprivation appeared to be not additive with that for leucine starvation.

DISCUSSION

In mammals, plasma concentrations of amino acids are affected by nutritional or pathological conditions. The experiments reported in this paper were designed to investigate the role of amino acids in the control of gene expression. A study performed by Marten *et al*. (12) showed that in a rat hepatoma cell line, removal of one amino acid in the culture medium

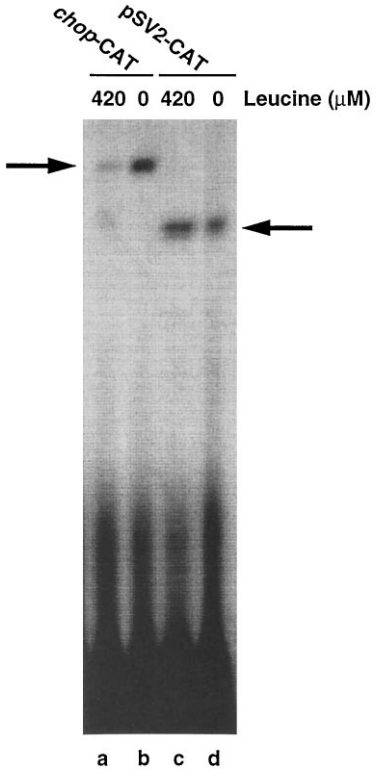

FIG. 6. **Regulation of CAT mRNA expression under the control of the *CHOP* promoter.** HeLa cells were transiently transfected with plasmid p*CHOP*-CAT or with plasmid pSV2CAT as described under "Materials and Methods"; 24 h after transfection, cells were incubated for 16 h in DMEM/F12 (420 μM) or in DMEM/F12 lacking leucine (0 μM) and harvested. Total cellular RNA was extracted, and 50 μg of each sample was analyzed for CAT mRNA expression by primer extension as described under "Materials and Methods." Each *arrow* indicates the CAT mRNA correctly initiated from the *CHOP* or the SV40 promoter.

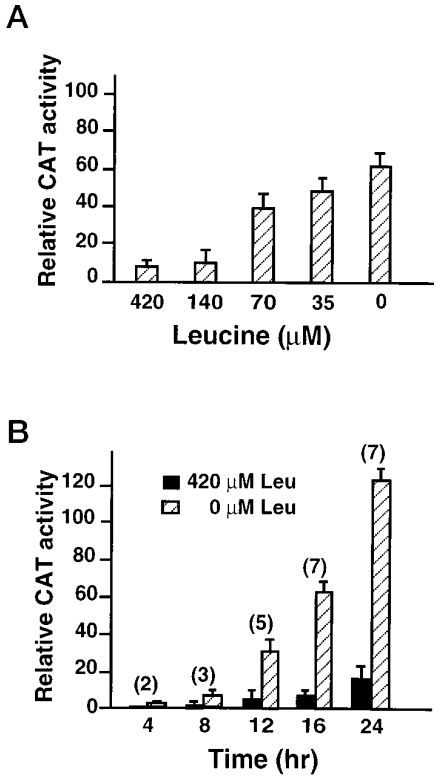

FIG. 7. **Characteristics of *CHOP* promoter response to leucine limitation.** *A*, effect of leucine concentration on *CHOP* promoter activity. HeLa cells were transiently transfected with p*CHOP*-CAT plasmid as described under "Materials and Methods"; 24 h after transfection, cells were incubated for 16 h in DMEM/F12 containing the indicated leucine concentrations. 420 μM leucine correspond to DMEM/F12 control medium. Relative CAT activities were determined as described under "Materials and Methods." *B*, kinetics of induction of *CHOP* promoter activity by leucine starvation. HeLa cells were transiently transfected with p*CHOP*-CAT plasmid as described previously. HeLa cells were incubated in DMEM/F12 (420 μM Leu) or in DMEM/F12 lacking leucine (0 μM Leu) and harvested for CAT activity determination after the indicated incubation times. The relative fold induction, defined as the ratio of the relative CAT activity of leucine-starved cells to unstarved cells, is indicated in *parentheses*.

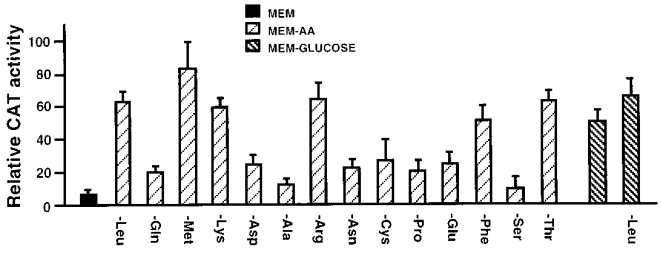

FIG. 8. **Effect of individual amino acid starvation on *CHOP* promoter activity.** HeLa cells were transiently transfected with p*CHOP*-CAT plasmid as described under "Materials and Methods"; 24 h after transfection, cells were incubated 16 h in MEM control medium (*MEM*), in MEM lacking one amino acid (*MEM-AA*), or in MEM lacking glucose (*MEM-GLUCOSE*) and harvested for CAT activity determination.

induced an increase in the expression of several genes. Among these genes, *CHOP* expression exhibited the greatest induction in response to amino acid starvation. Nevertheless, molecular mechanisms involved in the regulation of *CHOP* mRNA expression have not been elucidated to date. To understand the regulation of gene expression by amino acids at a molecular level, we have studied the regulation of *CHOP* expression in response to leucine limitation.

The main effect of amino acid limitation on cellular function is the inhibition of protein synthesis. We show that low leucine concentrations (35 and 70 μM) can induce *CHOP* expression but do not significantly inhibit total protein synthesis. However, this does not preclude the possibility that low leucine concentrations could affect the synthesis of particular proteins. These findings demonstrate that the regulation of *CHOP* expression by amino acid limitation is not a consequence of a cellular stress due to protein synthesis inhibition.

Since no general accumulation of mRNAs in amino acid-starved cells has been observed, mammalian cells must have a specific mechanism(s) that enables them to alter one specific pattern of gene expression in response to amino acid deprivation. Accumulation of asparagine synthetase, c-*myc*, c-*jun*, and c-*fos* mRNA have been reported to be induced transcriptionally and/or post-transcriptionally by amino acid starvation (8, 43, 44). We show that regulation of *CHOP* expression by leucine limitation has both transcriptional and post-transcriptional components. Our results clearly establish that the stability of *CHOP* mRNA is very low in the presence of leucine and is markedly increased in the absence of leucine. However, the mechanisms affecting *CHOP* mRNA stability in leucine-starved cells remain to be characterized. Furthermore, the induction of *CHOP* mRNA expression is sensitive to cycloheximide treatment suggesting that signaling pathways activated by leucine starvation involve synthesis of essential regulatory protein(s). We also show that starvation of other amino acids like lysine, methionine, arginine, phenylalanine, or threonine increases strongly *CHOP* promoter activity. These results suggest that gene regulation by leucine may be an example of a more general regulatory mechanism by which *CHOP* expres-

sion would be controlled by the levels of amino acids. In yeast, the general control response to amino acid starvation is mediated through translational control of the positive transcription factor GCN4 which in turn modulates expression of numerous genes (2, 45). Our results are in agreement with the existence in mammal cells of such a regulatory protein(s) involved in a general regulatory mechanism of gene expression by amino acid starvation. This hypothesis remains to be demonstrated. However, it is also possible, as suggested by Wang *et al*. (39), that what is being sensed is not the level of amino acids as such but rather some perturbation that arises when amino acid levels become limiting, for example the synthesis of abnormal proteins.

Our present results show that the 5′-flanking region of the human *CHOP* gene contains *cis* elements involved in the regulation of the *CHOP* transcription by leucine starvation. The promoter of the human asparagine synthetase gene has been shown to contain a 7-base pair region (5′-CATGATG-3′), designated amino acid response element (AARE), which mediates the transcriptional activation of the gene in response to amino acid starvation (9). Sequence analysis indicates that the *CHOP* promoter region contains several sequences homologous to the AARE, but their functional role remains to be demonstrated. Moreover, numerous regulatory elements that are likely to function in controlling the expression of this gene in response to cellular stress have been identified (31). Promoter deletion analyses have shown that several *cis* elements are involved in transcriptional activation of *CHOP* by UV irradiation or oxidant treatment (46). However, the *cis* elements involved in *CHOP* regulation by amino acid limitation remain to be identified.

The CHOP protein has been shown to heterodimerize with members of the C/EBP family (23). McKnight *et al*. (17) have hypothesized that C/EBP transcription factor family could play an important role in the control of energy metabolism. Through its interaction with C/EBPs, CHOP may participate in the regulation of downstream effector gene transcription during cellular response to amino acid limitation. It has been reported that C/EBP is involved in the transcriptional regulation of the carbamoyl-phosphate synthetase gene and two other urea cycle enzyme genes (47–49). Therefore, *CHOP* could play a crucial role in the regulation of nitrogen metabolism under amino acid control, although the cause and effect relationships have to be demonstrated.

In mammals, the plasma concentration of free amino acids shows striking alterations according to the nutritional or pathological conditions. For example, blood amino acid concentrations drop in animals fed with a low protein diet or starved (27, 50). Under such extreme nutritional conditions, cells could undergo a limitation for essential amino acids. Indeed, Strauss *et al*. (11) have hypothesized that induction of *IGFBP-1* gene expression in the liver of protein-restricted animals may be partially explained by a limitation for essential amino acids. We show that *CHOP* induction by amino acid limitation can take place (i) in all human cell lines tested (HepG2, CaCo-2, HeLa cells) and (ii) at a leucine concentration (70 μM) in the range of those observed in the blood of protein-restricted animals. Therefore, leucine limitation related to those observed in nutritional situations may be a factor contributing to the induction of *CHOP* gene expression. Further work will be necessary to determine whether changes in blood amino acid concentrations could play an important role, in concert with hormones, in the modulation of gene expression.

Acknowledgments—We are grateful to Dr. N. J. Holbrook for providing *CHOP* cDNA and p*CHOP*-CAT plasmids. We thank Drs. K. Boulukos, P. Brachet, J. L. Couderc, J. P. Jost, S. Mordier, and P. Pognonec for critically reading the manuscript and for helpful discussions. We also thank Y. Liu for the technical help in the nuclear run-on experiments.

REFERENCES

1. Kolter, R., and Yanofsky, C. (1982) *Annu. Rev. Genet.* **16,** 113–134
2. Hinnebusch, A. G. (1988) *Microbiol. Rev.* **52,** 248–273
3. Towle, H. C. (1995) *J. Biol. Chem.* **270,** 23235–23238
4. Vaulont, S., and Kahn, A. (1994) *FASEB J.* **8,** 28–35
5. Girard, J., Perdereau, D., Foufelle, F., Prip-Buus, C., and Ferré, P. (1994) *FASEB J.* **8,** 36–42
6. Clarke, S., and Abraham, S. (1992) *FASEB J.* **6,** 3146–3152
7. Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993) *Cell* **72,** 19–28
8. Pohjanpelto, P., and Hölttä, E. (1990) *Mol. Cell. Biol.* **10,** 5814–5821
9. Guerrini, L., Gong, S. S., Mangasarian, K., and Basilico, C. (1993) *Mol. Cell. Biol.* **13,** 3202–3212
10. Laine, R. O., Shay, N. F., and Kilberg, M. S. (1994) *J. Biol. Chem.* **269,** 9693–9697
11. Straus, D. S., Burke, E. J., and Marten, N. W. (1993) *Endocrinology* **132,** 1090–1100
12. Marten, N. W., Burke, E. J., Hayden, J. M., and Straus, D. S. (1994) *FASEB J.* **8,** 538–544
13. Luethy, J. D., and Holbrook, N. J. (1992) *Cancer Res.* **52,** 5–10
14. Bartlett, J. D., Luethy, J. D., Carlson, S. G., Sollot, S. J., and Holbrook, N. J. (1992) *J. Biol. Chem.* **267,** 20465–20470
15. Carlson, S. G., Fawcett, T. W., Bartlett, J. D., Bernier, M., and Holbrook, N. J. (1993) *Mol. Cell. Biol.* **13,** 4736–4744
16. Sylvester, S. L., ap Rhys, C. M. J., Luethy-Martindale, J., and Holbrook, N. J. (1994) *J. Biol. Chem.* **269,** 20119–20125
17. McKnight, S., Lane, M. D., and Gluecksohn-Waelsch, S. (1989) *Genes Dev.* **3,** 2021–2024
18. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* **5,** 1538–1552
19. Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H., and McKnight, S. L. (1989) *Genes Dev.* **3,** 1146–1156
20. Umek, R. M., Friedman, A. D., and McKnight, S. L. (1991) *Science* **251,** 288–292
21. Ron, D., and Habener, J. F. (1992) *Genes Dev.* **6,** 439–453
22. Ubeda, M., Wang, X. Z., Zinszner, H., Wu, I., Habener, J. F., and Ron, D. (1996) *Mol. Cell. Biol.* **16,** 1479–1489
23. Fawcett, T. W., Eastman, H. B., Martindale, J. L., and Holbrook, N. J. (1996) *J. Biol. Chem.* **271,** 14285–14289
24. Barone, M. V., Crozat, A. Y., Tabaee, A., Philipson, L., and Ron, D. (1994) *Genes Dev.* **8,** 453–464
25. Zinszner, H., Albalat, R., and Ron, D. (1994) *Genes Dev.* **8,** 2513–2526
26. Batchvarova, N., Wang, X. Z., and Ron, D. (1995) *EMBO J.* **14,** 4654–4661
27. Fafournoux, P., Remesy, C., and Demigne, C. (1990) *Am. J. Physiol.* **259,** E614–E625
28. Cynober, L. (1989) *J. Parenter. Enteral. Nutr.* **13,** 196–205
29. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., p.7.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Park, J. S., Luethy, J. D., Wang, M. G., Fargnoli, J., Fornace, A. J., Jr., McBride, O. W., and Holbrook, N. J. (1992) *Gene* (*Amst.*) **116,** 259–267
32. Davis, L. G., Dibner, M. D., and Battey, J. (1986) *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., Inc., New York
33. Gorman, C., Moffat, F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051
34. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
35. Hall, C. V., Jacob, P. E., Ringold, M., and Lee, F. (1983) *J. Mol. Appl. Genet.* **2,** 101–109
36. Trienzenberg, S. J. (1992) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G., Smith, J. A., and Struhl, K., eds) pp. 4.8.1–4.8.5, John Wiley & Sons, New York
37. Liu, Y., Sun, L., and Jost, J. P. (1996) *Nucleic Acids Res.* **24,** 2718–2722
38. Vincent, S., Marty, L., and Fort, P. (1993) *Nucleic Acids Res.* **21,** 1498
39. Wang, X.-Z., Lawson, B., Brewer, J. W., Zinszner, H., Sanjay, A., Mi, L.-J., Boorstein, R., Kreibich, G., Hendershot, L. M., and Ron, D. (1996) *Mol. Cell. Biol.* **16,** 4273–4280
40. Crozat, A. Y., Aman, P., Mandahl, N., and Ron, D. (1993) *Nature* **363,** 640–644
41. Kilberg, M. S., Stevens, B. R., and Novak, D. A. (1993) *Annu. Rev. Nutr.* **13,** 137–165
42. McGivan, J. D., and Pastor-Anglada, M. (1994) *Biochem. J.* **299,** 321–334
43. Gong, S. S., Guerrini, L., and Basilico, C. (1991) *Mol. Cell. Biol.* **11,** 6059–6066
44. Hutson, R. G., and Kilberg, M. S. (1994) *Biochem. J.* **304,** 745–750
45. Hope, I. A., and Struhl, K. (1985) *Cell* **43,** 177–188
46. Guyton, K. Z., Xu, Q., and Holbrook, N. J. (1996) *Biochem. J.* **314,** 547–554
47. Takiguchi, M., and Mori, M. (1991) *J. Biol. Chem.* **266,** 9186–9193
48. Lagacé, M., Goping, I. S., Mueller, C. R., Lazzaro, M., and Shore, G. C. (1992) *Gene* (*Amst.*) **118,** 231–238
49. Murakami, T., Nishiyori, A., Takiguchi, M., and Mori, M. (1990) *Mol. Cell. Biol.* **10,** 1180–1191
50. Nair, K. S., Woolf, P. D., Welle, S. L., and Matthews, D. E. (1987) *Am. J. Clin. Nutr.* **46,** 557–562