Amino acid limitation regulates gene expression

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In mammals, the plasma concentration of amino acids is affected by nutritional or pathological conditions. For example, an alteration in the amino acid profile has been reported when there is a deficiency of any one or more of the essential amino acids, a dietary imbalance of amino acids, or an insufficient intake of protein. We examined the role of amino acid limitation in regulating mammalian gene expression. Depletion of arginine, cystine and all essential amino acids leads to induction of insulin-like growth factor-binding protein-1 (*IGFBP-1*) mRNA and protein expression in a dose-dependent manner. Moreover, exposure of HepG2 cells to amino acids at a concentration reproducing the amino acid concentration found in portal blood of rats fed on a low-protein diet leads to a significantly higher ($P < 0{\cdot}0002$) expression of IGFBP-1. Using CCAAT/enhancer-binding protein homologous protein (*CHOP*) induction by leucine deprivation as a model, we have characterized the molecular mechanisms involved in the regulation of gene expression by amino acids. We have shown that leucine limitation leads to induction of *CHOP* mRNA and protein. Elevated mRNA levels result from both an increase in the rate of *CHOP* transcription and an increase in mRNA stability. We have characterized two elements of the *CHOP* gene that are essential to the transcriptional activation produced by an amino acid limitation. These findings demonstrate that an amino acid limitation, as occurs during dietary protein deficiency, can induce gene expression. Thus, amino acids by themselves can play, in concert with hormones, an important role in the control of gene expression.

Résumé

Chez les mammifères, la concentration plasmatique d'acides aminés peut varier selon les conditions nutritionnelles ou pathologiques. Par exemple, une déficience en un (ou plusieurs) acide(s) aminé(s) essentiel(s), un régime déséquilibré ou une restriction protéique vont entraîner une altération du profil plasmatique en acides aminés. Une sous-nutrition protéique a également pour conséquence un arrêt de la croissance dû en partie à une baisse de la concentration d'IGF-1 et à une très forte augmentation d'IGFBP-1 plasmatique. Nous avons montré qu'une déplétion en acides aminés essentiels ainsi qu'en arginine et cystine entraîne une forte induction de l'expression d'IGFBP-1 par des hepatocytes isolés ainsi que par des cellules d'hépatome de foie (HepG2) en culture. De plus, le traitement de cellules HepG2 par des concentrations d'acides aminés reproduisant celles trouvées dans le sang afférent au foie de rats nourris avec un régime pauvre en protéine conduit à une induction significative d'IGFBP-1. En utilisant comme modèle l'induction du gène *CHOP* par une carence en leucine nous avons caractérisé les mécanismes moléculaires impliqués dans la régulation de l'expression des gènes par les acides aminés. Nous avons montré que l'élévation du messager de *CHOP* en réponse à une carence en leucine est due à une augmentation de la transcription ainsi qu'à une stabilisation du messager. Nous avons également caractérisé deux éléments du gène *CHOP* essentiels à l'activation de la transcription consécutive à une limitation en acide aminé. Ces résultats montrent qu'une limitation en acides aminés similaire à celle observée lors d'un régime pauvre en protéine peut induire l'expression de gènes cibles. Les acides aminés peuvent donc par eux mêmes et de concert avec les hormones, jouer un rôle important dans le contrôle de l'expression des gènes.

Gene expression: Amino acids: Insulin-like growth factor-binding protein-1

Abbreviations: CAT, chloramphenicol acyltransferase; C/EBP, CCAAT/enhancer-binding protein; *CHOP*, C/EBP homologous protein; IGF, insulin-like growth factor; IGFBP-1, IGF-binding protein-1; LUC, luciferase; p*CHOP*, *CHOP* promotor.

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All 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 metabolize quickly various molecules based on their availability in the external medium. However, much less is known about the response of multicellular organisms to nutrient variations. The control of gene expression differs in many aspects from that operating in single-cell organisms, and involves complex interactions of hormonal and neuronal factors. Although not as widely appreciated, nutritional and metabolic signals play an important role in controlling gene expression in multicellular organisms. It has been shown that major (carbohydrates, fatty acids, and sterols) or minor (minerals and vitamins) dietary constituents participate in the regulation of gene expression in response to nutritional changes (for reviews, see Girard *et al*. 1994; Gurney *et al*. 1994; Vaulont & Kahn, 1994; Towle, 1995). There is considerably less information available concerning the control of mammalian gene expression by amino acids (Kilberg *et al*. 1994). However, the plasma concentration of free amino acids shows striking alterations according to the nutritional or pathological conditions. The current investigation examines the regulation of two genes, insulin-like growth factor-binding protein-1 (*IGFBP-1*) and CCAAT/enhancer-binding protein (C/EBP) homologous protein (*CHOP*) by amino acids. More particularly, we sought to determine (1) whether a change in blood amino acid concentrations following a change in the nutritional status leads to a modification of IGFBP-1 expression level, and (2) the molecular mechanisms involved in CHOP regulation by a leucine limitation.

Regulation of insulin-like growth factor-binding protein-1 by amino acids limitation

An example of amino acid limitation: protein undernutrition

Protein undernutrition has its most devastating consequences during growth. Deficiency of any one or more of the essential amino acids or a deficient protein intake is characterized by negative N balance, weight loss and impaired growth in infants and children (kwashiorkor; Coward & Lunn, 1981).

Growth is controlled by the complex interaction of genetic, hormonal and nutritional factors. A large part of this control is due to growth hormone and to the insulin-like growth factors (IGF)-I and -II (Baker *et al*. 1993; Binoux, 1995). The biological activities of the IGF are modulated by the IGFBP that specifically bind IGF-I and IGF-II (Walton *et al*. 1995; for review, see Lee *et al*. 1993). Of the six IGFBP, IGFBP-1 is the only one that displays rapid dynamic regulation *in vivo*, with serum levels varying 10-fold or more in relation to meals. Plasma IGFBP-1 is increased by fasting (Busby *et al*. 1988; Cotterill *et al*. 1988), malnutrition (Straus & Takemoto 1990; Donovan *et al*. 1991) and diabetes (Brismar *et al*. 1988; Suikkari *et al*. 1988). IGFBP-1 is mainly synthesized by the liver, and *in vivo* its expression is mainly controlled by insulin, growth hormone and glucose. Using IGFBP-1 administration in the rat (Lewitt *et al*. 1991) or in transgenic mice overexpressing hepatic IGFBP-1 (Rajkumar *et al*. 1995, 1996; Gay *et al*. 1997), it was shown that permanent hepatic expression of IGFBP-1 leads to growth retardation during both ante- and postnatal periods and impaired development of organs such as the brain.

Growth defects associated with protein malnutrition take place in the presence of normal or elevated serum levels of growth hormone (Vance *et al*. 1992). However, the circulating level of IGF-I is decreased whereas IGFBP-1 expression is strongly increased, suggesting that IGF-I and IGFBP-1 are differentially regulated and may play an important role in the nutritional regulation of growth (Straus, 1994; Kita *et al*. 1996). Straus *et al*. (1993) have reported a higher level of IGFBP-1 expression in protein-restricted animals than in starved animals. This difference cannot be explained by a variation in the plasma level of glucose, insulin or growth hormone. These authors suggested that other additional metabolic factors could be involved in *IGFBP-1* gene regulation. In the present report, we investigate whether an amino acid limitation such as occurs in protein-restricted animals can regulate IGFBP-1 expression.

Induction of insulin-like growth factor-binding protein-1 mRNA and protein expression by amino acid limitation

To understand the role of amino acids in IGFBP-1 expression, we have studied the effect of leucine limitation on *IGFBP-1* mRNA and protein content in a human hepatoma cell line HepG2. We chose leucine because: (1) leucine is an essential amino acid that is poorly utilized by the cells during a 16 h incubation period (data not shown); (2) leucine, which is transported by system L (Palacin *et al*. 1998), is rapidly equilibrated through the cell membrane. Fig. 1 shows that *IGFBP-1* mRNA and protein levels are

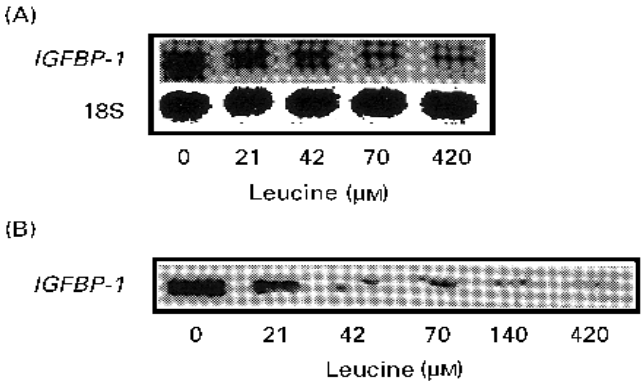

Fig. 1. Effect of leucine concentration on the expression of insulin-like growth factor-binding protein-1 (*IGFBP-1*) mRNA and protein in hepatoma cell line. HepG2 cells were incubated for 16 h in DMEM/F12 medium (Sigma, St Louis, MO, USA) containing the indicated leucine concentration. (A) Total RNA was extracted, and Northern blot analysis was performed. The blot was hybridized with a probe corresponding to human *IGFBP-1*. The same membrane was rehybridized with an 18S probe to normalize for RNA loading. (B) A 15 μl sample of culture medium was analysed for the presence of IGFBP-1 protein. Western blotting was performed using a rabbit polyclonal anti-IGFBP-1 antibody.

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very low in control cells, and increase dramatically when the leucine concentration is decreased in the culture medium. Similar results were obtained using primary culture of isolated rats hepatocytes (Jousse *et al*. 1998), showing that amino acid regulation of IGFBP-1 is not confined to hepatoma cells and occurs in well-differentiated isolated hepatocytes.

To characterize the role of amino acids in the control of IGFBP-1 expression we have tested the effects of individual amino acid depletion on hepatic *IGFBP-1* mRNA (results not shown). Depletion of arginine, cystine and all essential amino acids has a marked effect on *IGFBP-1* mRNA level in a human hepatoma cell line HepG2 (Jousse *et al*. 1998). It is noticeable that amino acids whose concentrations are affected by the nutritional status (Fafournoux *et al*. 1990) are those which play a major role in the control of IGFBP-1.

A change in nutritional status leads to a modification in blood concentration of most amino acids. However, the variation in concentration differs for individual amino acids. In order to determine whether a change in amino acid concentration corresponding to a change in nutritional status can modify IGFBP-1 expression, we reconstituted a culture medium reproducing the amino acids concentrations found in the portal blood of rats submitted to various diets (Fig. 2). Amino acid concentrations reproducing those of the portal blood of animals fed on a low-protein diet led to a significantly ($P < 0{\cdot}0002$) higher expression of IGFBP-1. These findings show that amino acid concentrations reproducing nutritional situations can significantly modify *IGFBP-1* gene expression.

In human subjects plasma amino acids profiles have been shown to be altered in the case of malnutrition. For example, alterations have been reported when there is a deficiency of any one or more of the essential amino acids, a dietary imbalance of amino acids, or a deficient intake of protein. Moreover, malnutrition is often associated with high levels of infection and infestation. The pattern of amino acids in the plasma of malnourished subjects is also influenced by the effects of infection and loss of appetite (Jackson & Grimble, 1990). In such a situation, the plasma concentrations of certain essential amino acids can be dramatically lowered. For example, leucine and methionine concentrations are reduced to 20 and 5 uM respectively in the plasma of children affected by kwashiorkor (Grimble & Waterlow, 1970; Baertl *et al*. 1974). Thus, in the case of

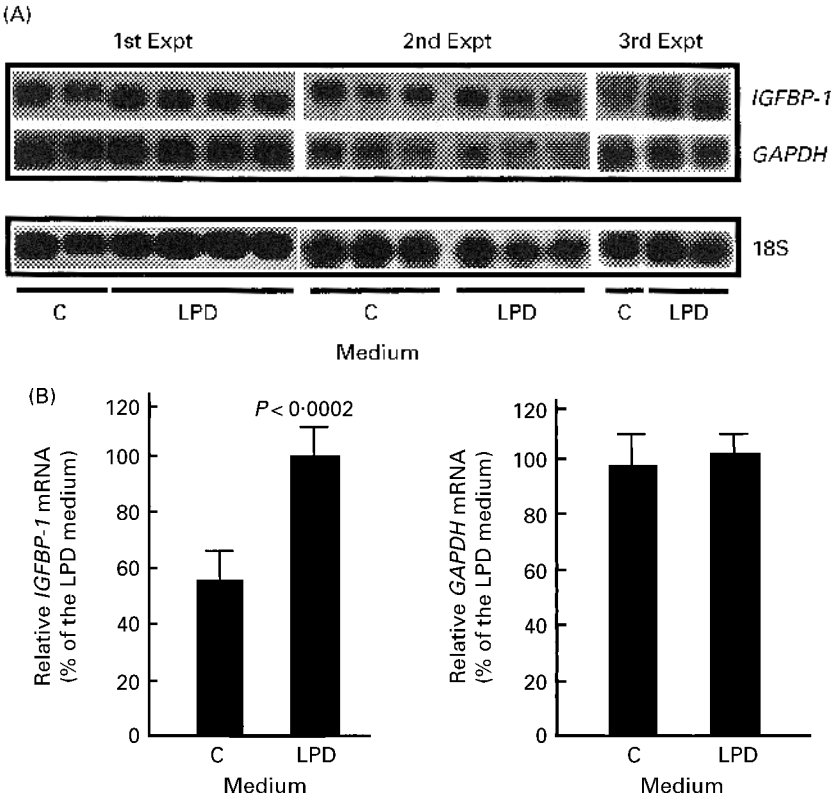

Fig. 2. Amino acid concentrations corresponding to physiological concentration modify the insulin-like growth factor-binding protein-1 (*IGFBP-1*) mRNA level. HepG2 cells were incubated for 16 h in a culture medium containing the liver afferent blood amino acid concentration corresponding to that of rats fed on a low-protein (LPD) or a standard (C) diet. Plasma afferent concentrations of amino acids are the average of data from the literature and from that of Jousse *et al.* (1998). Total RNA was extracted, and Northern blot analysis was performed (A). The blots were successively hybridized with a labelled probe corresponding to human *IGFBP-1*, glyceraldehyde-3-phosphate dehydrogenase (*EC* 1.2.1.12; *GAPDH*) and 18S. After quantification and normalization, results are given as percentages of IGFBP-1 or GAPDH expression in LPD medium (B). Values are means of three independent experiments and standard deviations represented by vertical bars.

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malnutrition amino acids by themselves play an important role in the regulation of IGFBP-1 expression, and in this fashion participate in the regulation of growth. The knowledge of the molecular basis of gene regulation by dietary protein intake is important with respect to growth regulation of young individuals living under conditions of restricted or excessive food intake.

Molecular mechanisms involved in gene regulation by an amino acid limitation

In mammalian cells, the current understanding of amino acid-dependent control of gene expression is limited. Our goal is to determine the molecular mechanisms involved in the regulation of gene expression by amino acid limitation. We focused on the regulation of one gene (*CHOP*) by one amino acid (leucine). We chose leucine for the reasons mentioned previously. We chose *CHOP* because: (1) the basal *CHOP* expression level is very low; (2) among the amino acid-regulated genes, *CHOP* expression exhibited the greatest induction in response to amino acid starvation; (3) *CHOP* is ubiquitous; (4) the *CHOP* promoter (p*CHOP*) has been cloned and characterized. *CHOP* (also known as *gadd153*) is a mammalian gene whose expression is induced in all tested cells by a wide variety of stresses and agents (Bartlett *et al*. 1992; Luethy & Holbrook, 1992; Carlson *et al*. 1993; Sylvester *et al*. 1994). *CHOP* induction is linked to the activation of an endoplasmic reticulum stress response, one that is presumably mediated by the accumulation of malfolded proteins in the endoplasmic reticulum (Wang *et al*. 1996). *CHOP* encodes a small nuclear protein related to the C/EBP family of transcription factors (Fornace *et al*. 1988). 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. By forming stable heterodimers with the members of the C/EBP family, CHOP protein can influence gene expression (Wang *et al*. 1998).

The induction of CHOP *expression by leucine deprivation involves both transcriptional and post-transcriptional mechanisms*

Recently, it was shown that leucine limitation induces *CHOP* expression in all cell lines tested (Bruhat *et al*. 1997). Leucine deprivation 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 deprivation (Fig. 3(A)). After 4 h of leucine deprivation the transcription of *CHOP* increased dramatically (21-fold), while the transcription of the *S26* ribosomal gene remained unchanged. To determine whether leucine deprivation can affect the half-life of *CHOP* mRNA, cells were first incubated for 16 h in a medium lacking leucine, 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. 3(B), addition of leucine resulted in a rapid decline in *CHOP* mRNA

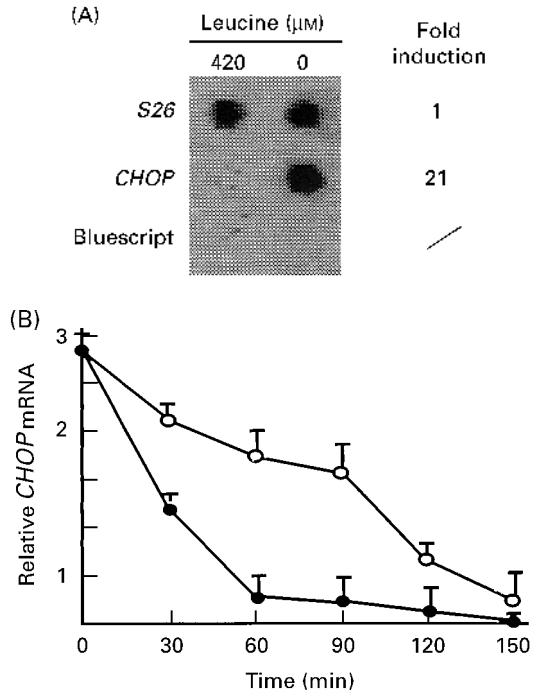

Fig. 3. Transcriptional and post-transcriptional regulation of CCAAT/enhancer-binding protein homologous protein (*CHOP*) by leucine deprivation. (A) Nuclear run-on analysis of *CHOP* transcription. HeLa cells were incubated for 4 h in DMEM/F12 control medium (Sigma, St Louis, MO, USA; 420 mM) or in DMEM/F12 medium lacking leucine (0 mM). ^{32}P-labelled RNA isolated from HeLa cells was hybridized to filter-bound DNA of ribosomal *S26*, *CHOP* and bluescript vector. The fold induction was determined as the ratio, mRNA expressed in leucine-deprivation : non-deprived mediums. (B) Effect of leucine deprivation on *CHOP* mRNA stability. HeLa cells were initially incubated for 16 h in DMEM/F12 medium lacking leucine. At this point (time 0), cells were incubated, in the presence of 4 mg actinomycin D/ml, either in DMEM/F12 medium (●—●) or in DMEM/F12 medium lacking leucine (○—○). Total RNA was extracted from each group of cells after the indicated incubation times and *CHOP* mRNA was analysed by Northern blotting.

levels. In deprived cells, the *CHOP* mRNA half-life was increased about 3-fold compared with cells incubated in the control medium. These findings indicate that leucine deprivation elevates *CHOP* mRNA levels both by increasing the rate of *CHOP* transcription and by enhancing the stability of *CHOP* mRNA.

Regulation of CHOP *promoter activity by leucine deprivation*

To analyse the role of p*CHOP* in transcription activation by leucine deprivation, a chimeric gene (p*CHOP*-luciferase; LUC) containing the 5′ flanking sequence from nucleotides –954 to +91 fused to the *LUC* gene was transiently transfected in HeLa cells under leucine-deprived or normal conditions. The data presented in Fig. 4 show that LUC activity expressed under the control of p*CHOP* was induced 7-fold by 16 h of leucine deprivation. These results give direct evidence that regulation of *CHOP* transcription by leucine

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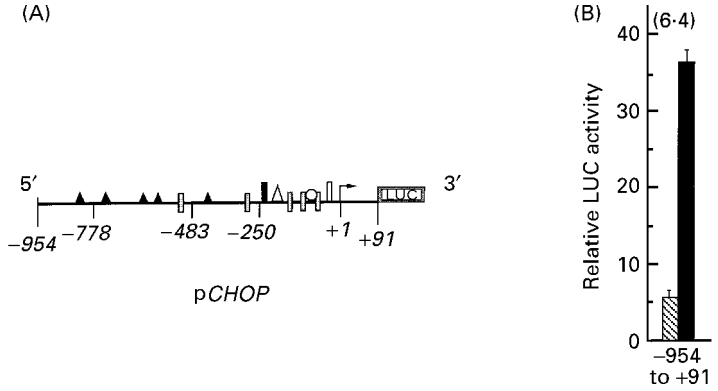

Fig. 4. Regulation of luciferase (LUC) activity under the control of the *CHOP* promoter in leucine-deprived cells. (A) The plasmid p*CHOP*-LUC corresponds to the human *CHOP* promoter (p*CHOP*) region from nucleotide −954 to +91 fused to the *LUC* gene. The putative binding site for the main transcription factors are represented: (▲), interleukin 6 response element; (▮), Sp-1; (△), c-ETS-1; (○), CCAAT box; (▮), AP-1; (▯) TATA box. (B) Hela cells were transiently transfected with plasmid p*CHOP*-LUC along with plasmid pCMV-βGAL where pCMV is cytomegalo virus promoter carrying the β-galactosidase (*EC* 3.2.1.23; β*GAL*) gene. At 24 h after transfection, cells were incubated for 16 h in DMEM/F12 medium (Sigma, St Louis, MO, USA; 420 μM; ▤) or in DMEM/F12 medium lacking leucine (■), then harvested for preparation of cells extracts, and LUC and βGAL activities were determined. Transfection efficiency was calibrated using βGAL activity. Results are given as LUC activity relative to βGAL activity). Values are means and standard deviations represented by vertical bars.

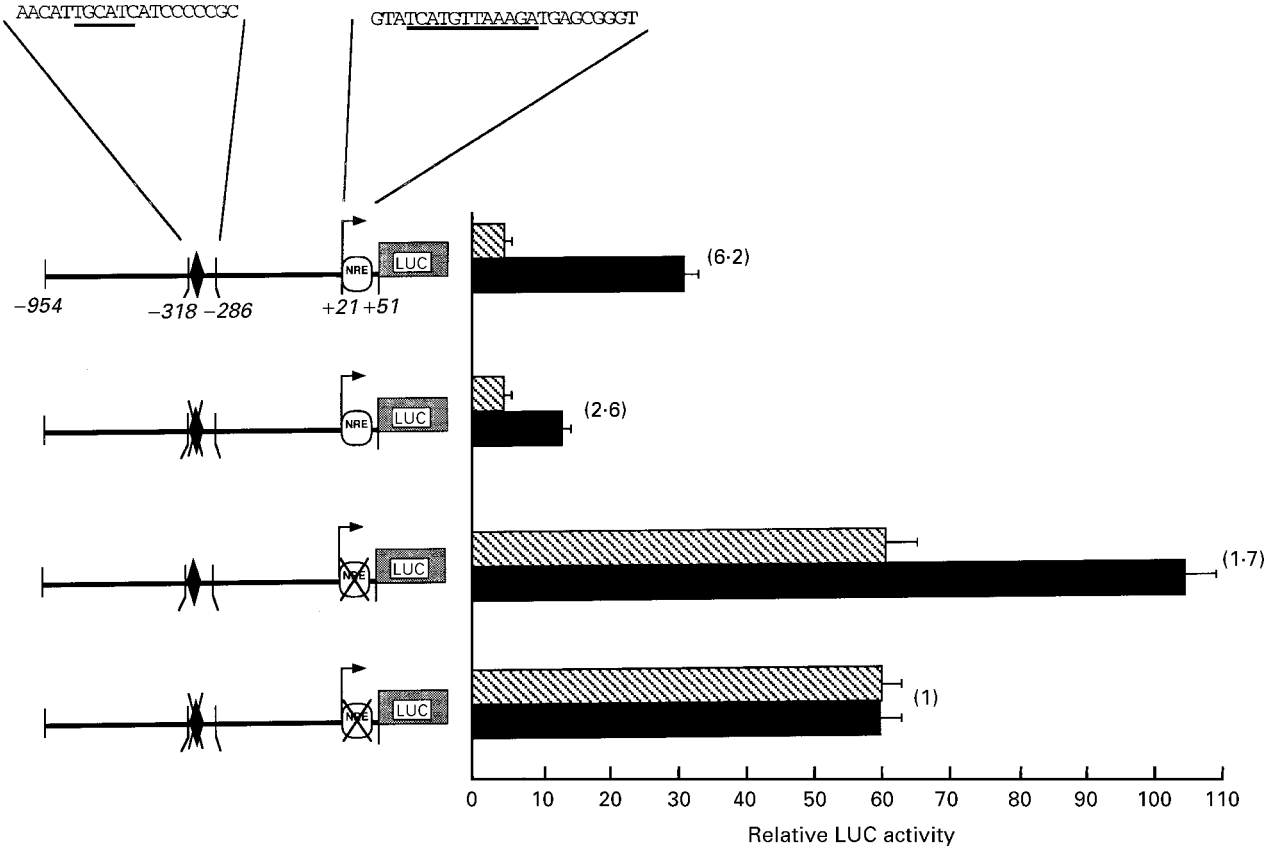

Fig. 5. Identification of the *CHOP* promoter region involved in the regulation by leucine limitation. Mutations of the *CHOP* promoter (p*CHOP*; in p*CHOP*-LUC, a chimeric gene containing the 5′ flanking sequence from nucleotides −954 to +91 fused to the luciferase (*LUC*) gene) were created by a polymerase chain reaction-based method. The mutated sequences are 'underlined'. The cells were transfected and then incubated for 16 h in a control medium (420 μM-leucine; ▨) or in a medium lacking leucine (▬), and then harvested for preparation of cell extracts, and LUC and β-galactosidase (*EC* 3.2.1.23) activities were determined. Tranfection efficiency was calibrated using β-galactosidase activity. Results are given as LUC activity relative to β-galactosidase activity. NRE, negative regulatory element. Values shown in parentheses are relative fold induction (LUC activity of leucine-deprived cells : LUC activity of control cells). Values are means and standard deviations represented by horizontal bars.

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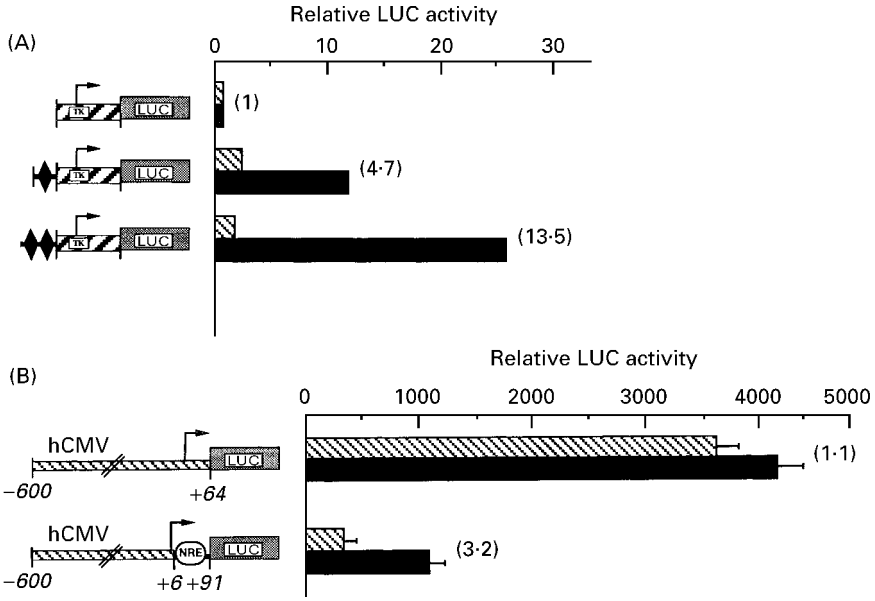

Fig. 6. Leucine deprivation activation of the amino acid-responsive elements placed in a heterologous promoter. (A) The *CHOP* amino acid-response element (◆) was placed in one or two copies upstream (–318, –286) of a minimum thymidine kinase (*EC* 2.7.1.21; TK) promoter driving the luciferase (LUC) construct. The cells were transfected and then incubated for 16 h in a control medium (420 μM-leucine; ▨) or in a medium lacking leucine (▬) and then harvested for preparation of cell extracts, and LUC and β-galactosidase (*EC* 3.2.1.23) activities were determined. Transfection efficiency was calibrated using β-galactosidase activity. Results are given as LUC activity relative to β-galactosidase activity. (B) The *CHOP* negative regulatory element (NRE) was placed downstream of a strong promoter (human cytomegalo virus (hCMV) promoter) driving LUC construct and transfected into HeLa cells. The cells were then incubated for 16 h in a control medium (420 μM-leucine; ▨) or in a medium lacking leucine (▬) and LUC activity was assayed as described previously. Values shown in parentheses are relative fold induction (LUC activity of leucine-deprived cell : LUC activity of control cells. Values are means and standard deviations represented by horizontal bars.

deprivation is mediated through the promoter sequence situated between nucleotides –954 to +91. Similarly, increased levels of LUC activity were also observed following transfection of p*CHOP*-CAT into other cell lines: HepG2 and Caco-2 cells (data not shown).

p*CHOP* contains numerous regulatory elements that are likely to function in controlling the expression of this gene in response to amino acid limitation. Serial deletions and mutations of p*CHOP* were undertaken to highlight regions responsive to amino acid deprivation (A Bruhat, C Jousse, S Blinet, M Ferrara and P Fafournoux, unpublished results). Two *cis* DNA elements which are important for amino acid regulation of p*CHOP* were identified. The first one is located in the region spanning nucleotides –313 to –295 and contains an amino acid response element. A mutation of this region markedly affects the amino acid regulation of p*CHOP* activity (Fig. 5). The second one is located downstream of the transcription initiation site of the *CHOP* gene. When this sequence is mutated the basal LUC activity is dramatically increased and the transcriptional activity of the promoter is still enhanced in response to leucine deprivation (Fig. 5). This element can be referred to as a negative regulatory element. When both regulatory elements are mutated p*CHOP* activity is no longer regulated by a leucine limitation (A Bruhat, C Jousse, S Blinet, M Ferrara and P Fafournoux, unpublished results).

In order to provide further evidence relating to the role of these two elements in the control of gene expression, we transferred the amino acid responsiveness to a heterologous promoter (Fig. 6). We inserted one or two copies of the amino acid response element of p*CHOP* into a minimum promoter sequence of the thymidine kinase (*EC* 2.7.1.21) gene. Fig. 6 shows that the *CHOP* amino acid response element sequence is enough to confer an amino acid regulation to the thymidine kinase promoter. We also tested the amino acid responsiveness of the *CHOP* negative regulatory element described previously. We inserted one copy of this sequence into the leader of the *LUC* gene driven by a strong promoter (cytomegalo virus) which is an early enhancer promoter region. As shown in Fig. 6, the negative regulatory element sequence dramatically decreases the LUC activity and confers an amino acid responsiveness to the cytomegalo virus promoter.

We have identified and characterized two elements of the *CHOP* gene essential to the transcriptional activation produced by an amino acid limitation. Further work will be required to identify precisely the *trans*-acting factor(s) involved in the regulation of the *CHOP* promoter as well as the mechanism of its activation by amino acid deprivation.

Our results represent the first demonstration that a shortage of amino acids, such as that occurring in protein-restricted animals, can control gene expression. Thus, amino acids by themselves can play, in concert with hormones, an important role in the control of gene expression. These studies will allow an understanding of the role of amino acids in the regulation of physiological functions (growth, proteolysis etc.). Defining the molecular steps by

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which the cellular concentration of individual amino acids can regulate gene expression will be an important contribution to our understanding of metabolite control in mammalian cells.

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