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Amino acid deprivation regulates the stress-inducible gene p8 via the GCN2/ATF4 pathway

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ABSTRACT

In mammals, the GCN2/ATF4 pathway has been described as the main pathway involved in the regulation of gene expression upon amino acid limitation. This regulation is notably conferred by the presence of a cis-element called Amino Acid Response Element (AARE) in the promoter of specific genes. *In vivo*, the notion of amino acid limitation is not limited to nutritional context, indeed several pathological situations are associated with alteration of endogenous amino acid availability. This is notably true in the context of tumour in which the alteration of the microenvironment can lead to a perturbation in nutrient availability. P8 is a small weakly folded multifunctional protein that is overexpressed in several kinds of cancers and whose expression is induced by different stresses. In this study we have demonstrated that amino acid starvation was also able to induce p8 expression. Moreover, we brought the evidence, *in vitro* and *in vivo*, that the GCN2/ATF4 pathway is involved in this regulation through the presence of an AARE in p8 promoter.

1. Introduction

All the organisms possess adaptative mechanisms to cope with changes in nutrient availability. In mammals, amino acid homeostasis presents two important features. Firstly, in adult human nine amino acids are essential since they cannot be synthesized *de novo* by the organism; secondly, there is no specific storage of amino acids. In consequence, if essential amino acids are not enough provided by diet, the organism has to tune finely amino acid homeostasis. In addition to a dietary deficiency, amino acid homeostasis can be affected by several pathological situations such as cancer, sepsis or trauma [1–3]. It exists different levels of adaptation to restore amino acid homeostasis i.e. exchanges between organs, modification of the flux of proteolysis and proteosynthesis. Another level of adaptation is the modification of gene expression. Indeed, it has been demonstrated that amino acids by themselves are able to regulate the expression of specific genes [4,5] such as amino acid transporters, enzymes or transcription factors.

Since several years the understanding of the molecular mechanisms involved in the regulation of gene expression by amino acid limitation in mammals has progressed [6–9]. One of the major pathways implies the kinase GCN2. This protein detects the accumulation of free tRNA which leads to the induction of its activity. The phosphorylation, by GCN2, of its substrate, the α subunit of the initiation faction eIF2, induces a global inhibition of protein synthesis but also an increase of the translation of specific mRNA such as the transcription factor ATF4 (activating transcription factor 4) due to the presence of uORF in its 5'UTR [10]. ATF4 is then able to bind amino acid response element (AARE) located in the promoter of specific genes and to induce their transcription [11]. The core sequence of the AARE is 5′-$\frac{A}{G}$TT$\frac{G}{T}$CATCA-3′ and according to the differences observed between genes, several transcription factors and cofactors (ATF2, C/EBPb, JDP2...) [8,12,13] are also involved positively or negatively in the regulation of the AARE dependent transcription. However several studies have determined the master role of ATF4 in the regulation of the AARE-dependent transcription [11,14,15]. So far, few genes have been described as possessing an AARE sequence in their promoters [15]. By promoter sequence analysis we have identified a putative AARE site in the promoter of the p8 gene. Previous studies have determined that this sequence was a C/EBP/ATF binding site [16,17].

P8, also known as nupr1, is a small protein of 82 amino acids that possesses notably a nuclear localization signal and a basic helix-loop helix motif. An architectural role of p8 has been notably suggested in the regulation of transcription [18]. P8 has been first described as a gene induced during the pancreatitis in rats [19]. It is described as stress inducible protein, indeed the expression of p8 mRNA has been shown to be induced by several kinds of cellular

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stresses such as, serum withdrawal or a stress of the endoplasmic reticulum (ER) [20,21]. Notably, it has been shown that an ER stress induces p8 expression in an ATF4-dependent manner [22]. The precise role of p8 is not well determined. According to the context, p8 has been shown to promote either cell survival or cell death. P8 being over-expressed in several kinds of tumours, the understanding of its regulation would be relevant in order to precise its function according to the cellular context [23].

It is noticeable that the tumour microenvironment may present an alteration of the vascularisation, leading to a deficit in blood supply, and as a consequence a deprivation of nutrients and notably amino acid. In consequence the tumour has to cope with this deficit in amino acid through the activation of adaptative mechanisms. Recently Ye et al. have demonstrated the importance of the GCN2/ATF4 pathway in the maintenance of metabolic homeostasis in tumour cells [24]. The impact of amino acid availability on p8 expression has never been studied, however it could represent an important feature in the context of the modification of the tumour microenvironment. Moreover it would also signify that p8 may have a role in other pathological or nutritional contexts where the amino acids availability is compromised. This study proposes to establish whether p8 is regulated by amino acid deficiency and to assess the role of the GCN2/ATF4 pathway in such regulation.

2. Material and methods

2.1. Ethics statement

Maintenance of the mice and experiments were conducted according to the guidelines formulated by the European Community for the use of experimental animals (L358-86/609/EEc) and were approved by the Institut National de la Recherche Agronomique (INRA-France). INRA animal facilities were approved by the french veterinary department (C634514).

2.2. Animal

Mice were maintained in our animal facility as previously described [25]. The experimental diets, control and leucine devoid, were identical to our previous study [25] The mice were acclimated to control diet for 7 days and to overnight starvation. On the morning of day of experiment, mice had free access to experimental diets for 2 h or 4 h until they were killed by pentobarbital overdose. Each animal experiment was repeated three times with four animals in each group.

2.3. Cell culture

MEFs and NIH-3T3 cells were grown in DMEM with 10% FCS at 37 °C with 5% CO_2. The media lacking leucine has been previously described [11].

2.4. Protein analysis

Protein extraction was performed with our standard lysis buffer (50 mM Tris, 25 mM beta-glycerophophate, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0,5% (v/v) triton X100, 1 mM DTT, 1 mM benzamidine, protease cocktail inhibitors (Sigma). SDS–PAGE were performed as described previously [11].

2.5. Plasmid constructions

2X-AARE-TK Luc and ATF4 plasmids have been previously described [6,11]. P8 promoter fragment (−1399 + 11) have been obtained from genomic mice DNA by PCR, primers containing appropriate restriction sites at their 5′ end. Forward primer: 5′-catcgcacgcgtcaaaggcaggttgatctctgag; Reverse primer: 5′-caagcgctcgagtcttgcctgtgtctccttgtctgaatgtctg. Amplified fragments were then cloned into the pGL3-basic reporter construct (Promega) by MluI and XhoI digestion.

The p8 mutated promoter has been obainetd by PCR using the previous construct as template.

The same forward primer than previously was used, the reverse primer contained the AARE mutated sequence and a part of the promoter containing a BstxI restriction site. Reverse primer: 5′cctaaaccacggggttggcccccgccctccaagccctgtctgatgcattctggccctttccc. The amplified fragment has been cloned in the previous construct after a MluI BstxI digestion.

2.6. DNA transfection and luciferase assay

Cells were transfected by the calcium phosphate coprecipitation method as described [6], 0.5 μg of luciferase plasmid and ATF4 plasmid were transfected into the cells with 0.05 μg of pCMV-βGal, a plasmid carrying the bacterial β-galactosidase gene fused to the human cytomegalovirus as an internal control. The total amount of plasmid DNA was adjusted to 1.05 μg by addition of the empty plasmid. Cells were then exposed to the precipitate for 16 h. Luciferase activity and β-Galactosidase activity were measured as described previously [11].

2.7. RNA extraction and reverse-transcription

Total RNA was prepared using an RNeasy mini kit (Qiagen) and treated with DNase I, Amp Grade (Invitrogen). RNA (0.5 μg) was reverse-transcribed with 100 U of Superscript II plus RNase H reverse transcriptase (Invitrogen) using 100 μM random hexamer primers.

2.8. Real time PCR

PCR was carried out using a CFX96 (Biorad) and iQ-SYBR green supermix. The following experimental protocol was used for all the primers: denaturation (95 °C for 10 min), amplification (repeated 45 times; 95 °C for 15 s, 60 °C for 5 s and 72 °C for 8 s). Primer used: ASNS-For 5′-tacaaccacaaggcgctaca and ASNS-Rev 5′-aagggcctgactccataggt-3′; p8-For 5′-ctagaggatgaagatggaatcctg and p8-REV 5′-gagttctggaacttggtcagca; batc-For 5′-tacagcttcaccaccacagc and bact-Rev 5′-aaggaaggctggaaaagagc.

2.9. Chromatin immunoprecipitation analysis (ChIP)

ChIP analysis was performed according to the protocol of Upstate Biotechnology, Inc. (Charlottesville, VA) with minor modifications as previously described [25]. Real-time PCR was performed as described. Primers used: p8-For 5′-gtgagtcctgtgagggagga and p8-Rev 5′- agccctgtctgatgcaatct.

3. Results

The first step of this study was to determine if leucine limitation induced the expression of p8 mRNA and if such induction was GCN2-dependent. GCN2 deficient mouse embryonic fibroblast (MEF) has been used to check this hypothesis. In GCN2+/+ cells, as expected, the consequences of leucine starvation were the phosphorylation of eIF2α and the induction of ATF4 protein level (Fig. 1A). Consistently with our previous data and those from the literature, in GCN2−/− cells the phosphorylation of eIF2a and the induction of ATF4 were abolished. Then, the level of p8 mRNA has been measured in GCN2+/+ and GCN2−/− cells which have been leucine starved for 2, 4 and 8 h. In parallel, the level of asparagine synthetase (ASNS) mRNA has been determined as a positive

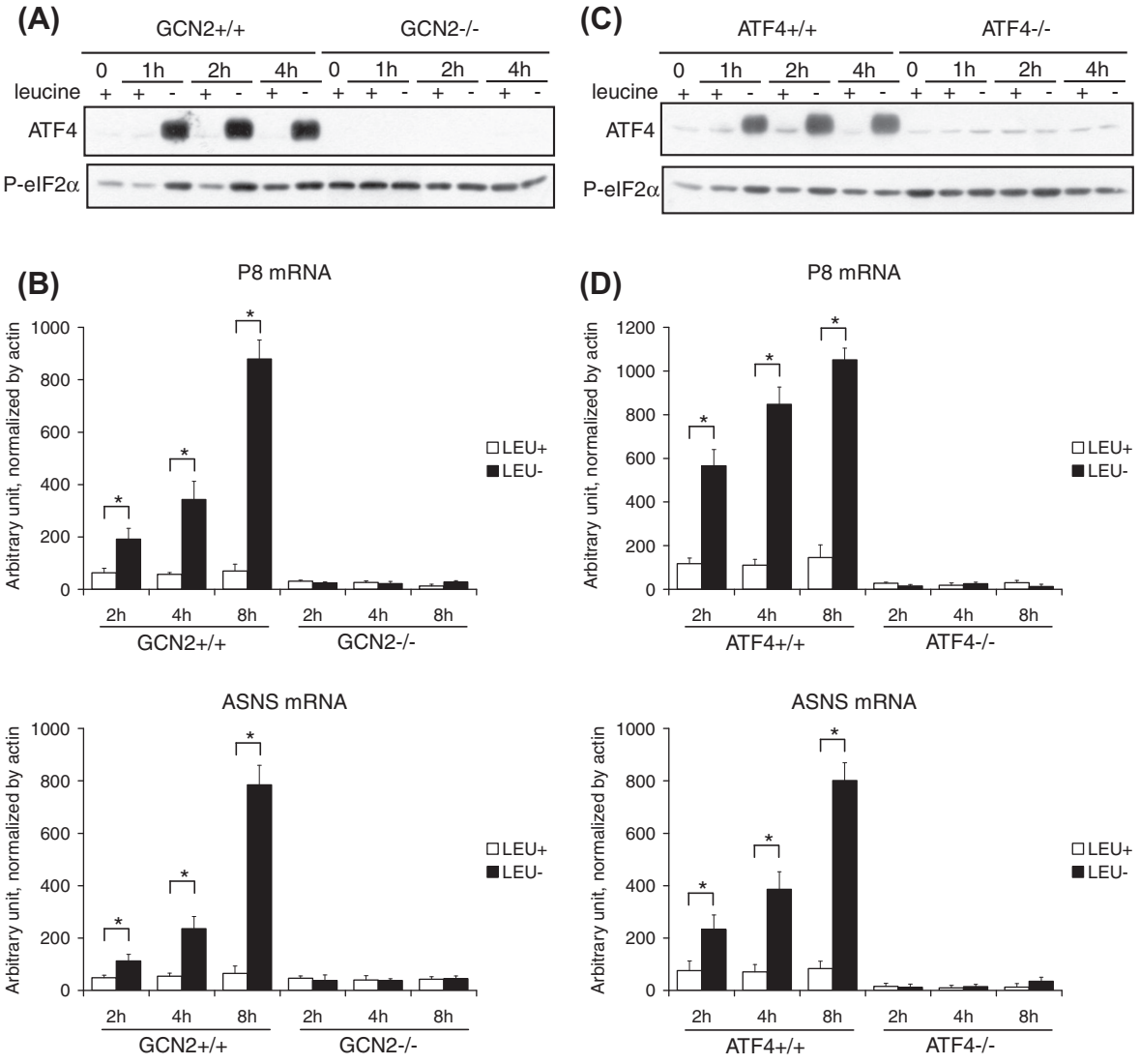

Fig. 1. Leucine starvation induces p8 mRNA expression in a GCN2/ATF4-dependent manner. MEFs cells (GCN2+/+, GCN2−/−, ATF4+/+, ATF4−/−) were cultured in DMEM/F12ham devoid or not of leucine during 1, 2, 4 and 8 h. (A) Western blot analysis of Pser51-eIF2α and ATF4 from total protein extract of control and leucine starved GCN2+/+ and GCN2−/− cells. (B) The mRNA levels of p8 and ASNS were measured in GCN2+/+ and GCN2−/− cells by real-time PCR, results were normalized by actin mRNA levels and were expressed in% of the time 0 h. (C) Same experiments than a, with ATF4+/+ and ATF4−/− cells. (D) Same experiments than b, with ATF4+/+ and ATF4−/− cells. Differences between control cells and leucine starved cells at each time point were assessed by Student's test. Bars with (∗) are significantly different from each other ($P < 0.05$).

control (Fig. 1B). It has been well described that leucine starvation induces ASNS expression in a GCN2/ ATF4 dependent-manner. Indeed, in GCN2+/+ cells the amount of ASNS mRNA was induced by the absence of leucine whereas it remained unaffected in GCN2−/− cells. In GCN2 +/+ cells, the amount of p8 mRNA was significantly increased since 2 h of starvation and an induction of 9-fold was observed after 8 h (Fig. 1B). This induction was not observed in leucine starved GCN2−/− cells. These results demonstrated that leucine starvation induced p8 expression in a GCN2-dependent manner.

In order to determine whether ATF4 was involved in the regulation of p8 mRNA during leucine starvation, we have performed the same experiments in ATF4 deficient MEF. The ATF4+/+ cells behaved in the same way than the GCN2+/+ in term of phosphorylation of eIF2a and ATF4 expression (Fig. 1C). In ATF4−/−, the phosphorylation of eIF2α was still observed, however due to the absence of ATF4 the induction of ASNS mRNA was abolished; the same result has been obtained for p8 mRNA (Fig. 1D). It appeared that the regulation of p8 mRNA by leucine starvation depended on the presence of ATF4.

This last result sustained the hypothesis that the C/EBP/ATF site present in p8 promoter is a functional AARE. Indeed, as illustrated in Fig. 2A the C/EBP/ATF site located between −111 and −103 is identical to the AARE sequence characterized in the CHOP promoter. A fragment of 1399 bases pairs of the mouse p8 promoter has been cloned in front of the luciferase reporter perform. A point mutation of the C/EBP/ATF sequence in the p8 promoter has been also performed. This point mutation, in the context of CHOP promoter, has been previously shown to abolish the responsiveness of the AARE sequence to leucine starvation [6] by disrupting ATF4 binding to the AARE [11]. We have used as a positive control a plasmid containing two copies of the CHOP AARE in front of a minimal promoter TK (2XAARE-TK). These constructions have been tested by transient transfection in NIH-3T3 cells. During leucine starvation an induction of the transcriptional activity of p8 promoter was observed (Fig. 2B). We obtained a similar result with the 2XAARE-TK construct. Moreover the mutation of the C/EBP/ATF sequence abolished totally the induction of the transcriptional activity. We have also co-transfected an ATF4 expression vector together with each construct. The over-expression of ATF4 has been

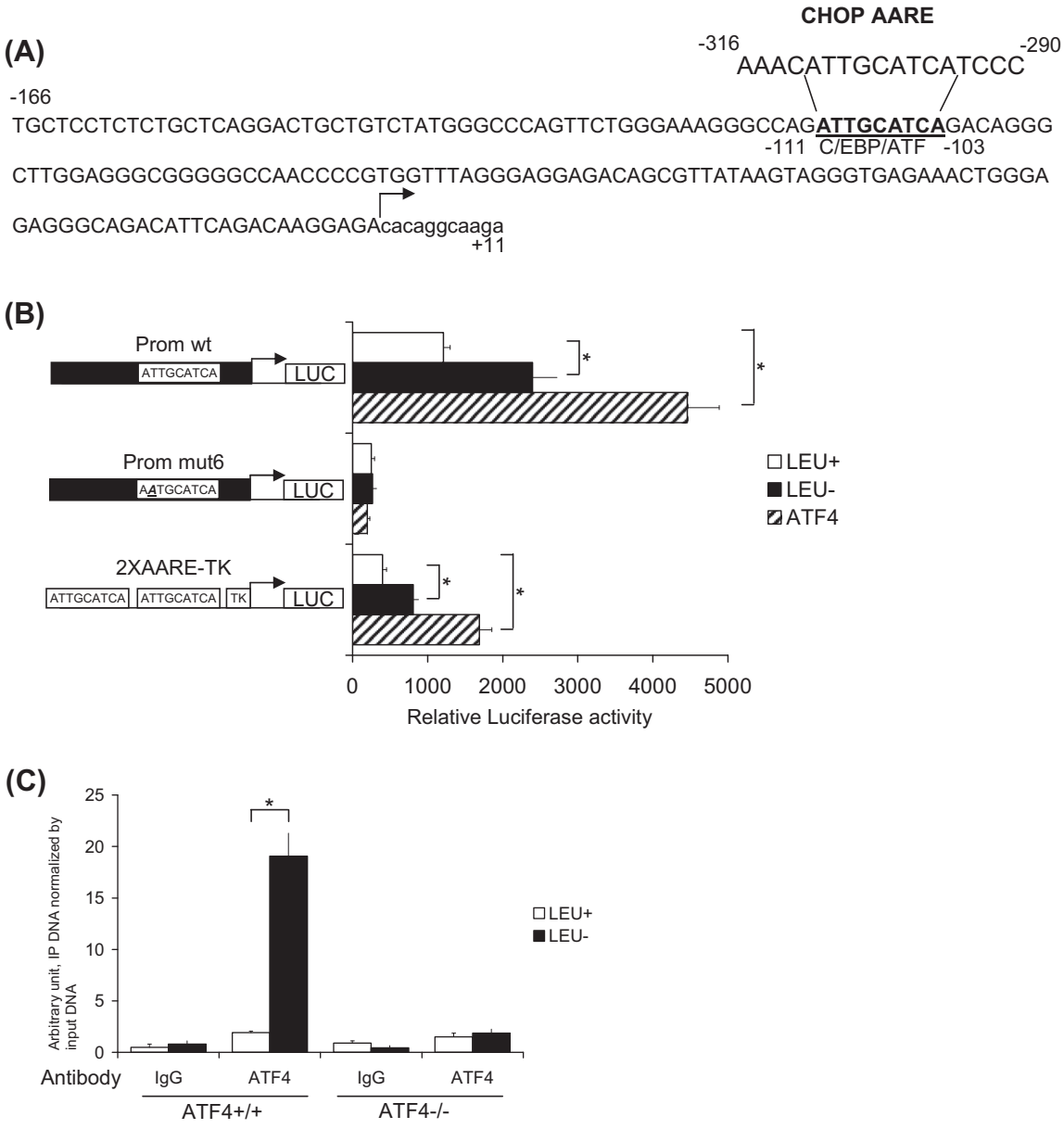

Fig. 2. The C/EBP-ATF site in p8 promoter is a functional AARE. (A) Representation of p8 promoter. The C/EBP-ATF site is identical to the CHOP AARE sequence. (B) NIH-3T3 cells were transfected with a plasmid encoding the luciferase gene driven either by: the wild-type promoter of p8 (prom-wt), the promoter of p8 mutated in the C/EBP/ATF sequence (prom mut6) or two copies of the CHOP-AARE and the minimal promoter of Thymidine-Kinase (2XAARE-TK). When indicated, the cells were also co-transfected with an expression vectors encoding ATF4. A plasmid pCMV-βGAL was used as an internal control. Twenty-four hours after the transfection the cells were cultured for 8 h in DMEM/F12ham devoid or not of leucine. Results were expressed as the ratio of the luciferase activity normalized by the beta-gal activity. c) MEF ATF4+/+ and ATF4−/− were cultured in DMEM/F12ham devoid or not of leucine for 2 h, ChIP analysis were performed using an antibody specific for ATF4 or a non-specific antibody control (IgG). The amount of PCR product obtained with immunoprecipitated DNA (IP) were normalized with the product obtained with input DNA. Differences between control cells, leucine starved cells or ATF4 transfected cells were assessed by Student's test. Bars with (∗) are significantly different from each other ($P < 0.05$).

shown to induce the AARE-dependent transcription. The induction observed with the 2XAARRE-TK confirmed this previous result. Concerning the p8 promoter, the over-expression of ATF4 induced the transcriptional activity of the wild type but has no effect on the mutated promoter (Fig. 2B). These experiments confirmed that the C/EBP/ATF site present in the promoter was responsible for the induction of the transcription during leucine starvation. In consequence, the p8 C/EBP/ATF site is a functional AARE sequence. To further confirm that the induction of transcription by ATF4 was a direct consequence of the binding of ATF4 to the AARE in the p8 promoter, Chromatin immuno-precipitaion (ChIP) experiment has been performed. ATF4−/− cells have been used as negative control. In ATF4+/+ cells, the binding of ATF4 on the sequence containing the AARE was found to be induced about 8-fold after 2 h of leucine starvation (Fig. 2C).

These results demonstrated *in vitro* that the GCN2/ATF4 pathway controls p8 expression in response to leucine starvation. However we wanted to assess whether this regulation could take place *in vivo,* in a nutritional context for which the activation of the GCN2/ATF4 pathway have been well described [25]. Mice were fed with a control diet or with a diet deficient in leucine which induced a rapid and important decrease of plasma leucinemia (data not shown). The level of p8 and ASNS mRNA has been measured in liver of wild-type and GCN2 knock-out mice. In wild type mice an induction of ASNS was observed 2 h following the consumption of the leucine deficient diet (Fig. 3A). Four hours after the beginning

of the meal consumption, for both p8 and ASNS mRNAs, an induction was observed. In GCN2 knock-out mice liver, no induction was observed for ASNS and p8 mRNA. For the last, we even observed a decrease of its amount following the consumption of the leucine deficient diet (Fig. 3A). The binding of ATF4 on p8 promoter *in vivo* has been checked by ChIP as described above. In wild type mice, the interaction of ATF4 with p8 promoter was confirmed whereas in absence of GCN2 this interaction disappeared (Fig. 3B). Together these results confirmed that the regulation of p8 expression takes place *in vivo* in a nutritional condition leading to a deficit in an essential amino acid.

4. Discussion

The concept of regulation of gene expression by amino acid in mammals is now well established. In the last decade, the GCN2/ATF4 pathway has emerged as the main pathway involved in the regulation of gene expression by amino acid limitation [26]. Importantly, it has been recently demonstrated that this signalling pathway, which have been well characterized *in vitro*, was also relevant *in vivo* in a context of a nutritional challenge [25]. However the number of genes for which a functional AARE has been characterized is still limited. Their identification represents an important step in order to have a better understanding in the role of the GCN2/ATF4 pathway in the adaptation to amino acid limitation.

In the present study, we have shown that p8 expression was induced by leucine starvation. The essential role of GCN2 in this regulation was confirmed by using GCN2−/− cells and mice. Our experiments have also demonstrated that ATF4 was necessary for the regulation of p8 expression during leucine starvation. Moreover, the study of p8 promoter and the ChIP experiments have confirmed that the C/EBP/ATF composite site was a functional AARE able to bind ATF4 during leucine starvation. These results were in agreement with previous studies which have demonstrated that ATF4 was able to activate p8 expression [22]. ATF4 translation is induced consecutively to the phosphorylation of eIF2α, this step being the initial event of the Integrated Stress Response (ISR). Indeed, eIF2α is phosphorylated by four different kinases: GCN2, PERK (activated by ER stress), PKR (activated by interferon and viral infection) and HRI (activated by heme deficiency). This phosphorylation event is going to coordinate a translational and transcriptional program, notably through ATF4, which adapt cell to a stress [27]. In consequence, p8 expression should be induced by any treatment that induces the ISR. Indeed, Salazar and coll have demonstrated that the eIF2α phosphorylation occurring during cannabinoïd treatment was responsible for the induction of p8 expression [21]. In this Study, the phosphorylation of eIF2α was proposed to be the consequence of an ER stress caused by ceramides accumulation following cannabinoïd treatment. As well, several drugs, which induce an ER stress, increase p8 expression in an ATF4-dependent manner [22]. Our present work enforces the notion that p8 is a part of the ISR and for the first time we demonstrated that one of the eIF2α kinase is involved in the regulation of p8 expression following a cellular stress. Due to the presence of an AARE, p8 can be considered as one of the early target of the transcriptional program of the ISR. In the context of cannabinoïd treatment, it has been proposed that p8 is involved in the induction of apoptosis and autophagy following an ER stress [21]. The

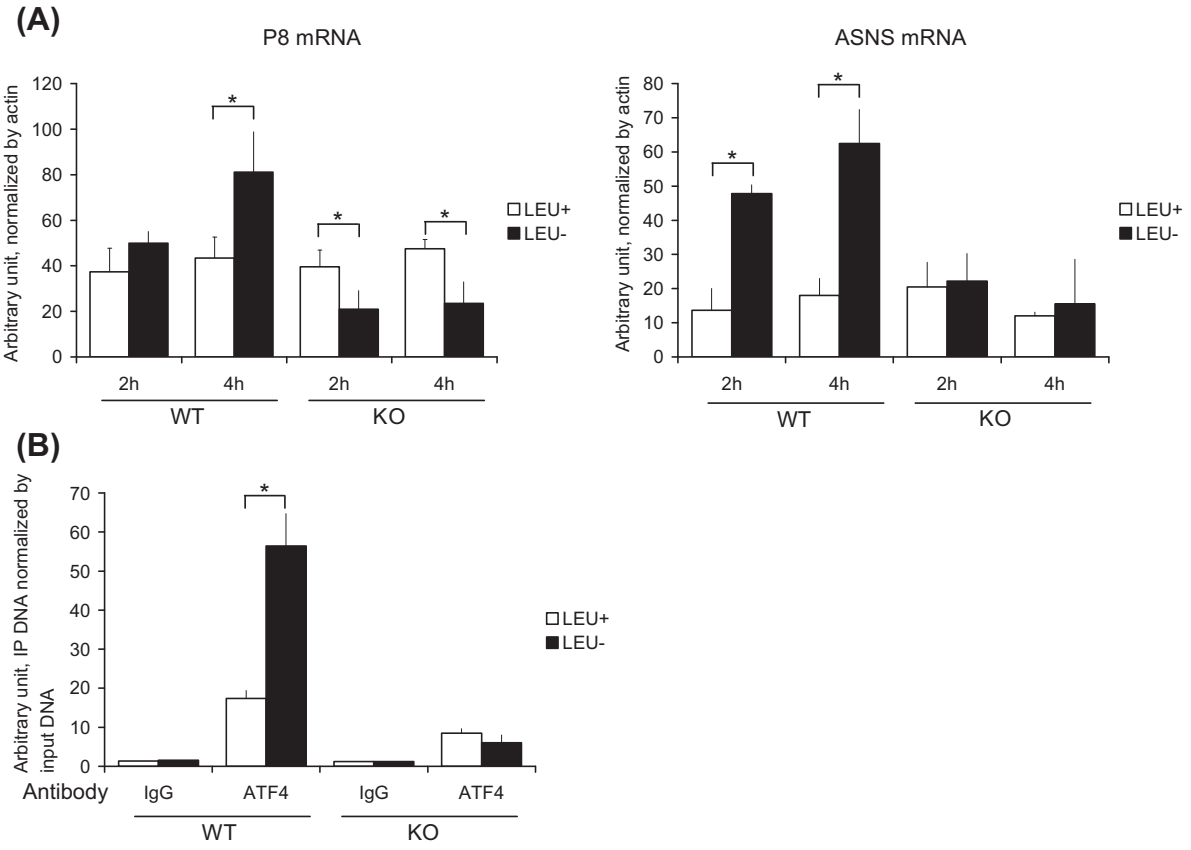

Fig. 3. The regulation of p8 by leucine availability occurs *in vivo* in mice liver. Overnight starved mice were fed for 2 and 4 h either with a control (Leu+) or a leucine-deficient (Leu-) diet. (A) The mRNA levels of p8 and ASNS were measured in total hepatic RNA by real time PCR, the results were normalized by actin mRNA levels and were expressed in% of the time 0 h. (B) ChIP analysis were performed on DNA from liver nuclear extract using an antibody specific for ATF4 or a non-specific antibody control (IgG). The amount of PCR product obtained with immunoprecipitated DNA (IP) were normalized with the product obtained with input DNA. Differences between mice fed with control diet and mice fed with leucine diet were assessed by Student's test. Bars with (∗) are significantly different from each other ($P < 0.05$).

authors have also demonstrated that p8 was involved in the proper induction of ATF4 and several ISR regulated gene such as trb3 or CHOP. Similarly, Jin et al. have shown that over-expression of p8 led to the increase of ATF4 expression and in consequence to the induction of CHOP and TRB3 expression [22]. However, it is not yet clear how p8 may regulate ATF4 expression, nevertheless this regulation could define a positive feedback in the context of the ATF4-dependent transcription. Further investigation will be necessary to elucidate this question.

Our work demonstrated that the GCN2 pathway was involved in the regulation of p8 through an AARE in the context of amino acid limitation. This finding extends the field of investigation concerning this small puzzling stress protein. It would be of interest to determine the role of p8 in the adaptation to amino acid limitation. It represents an interesting issue not only in term of nutrition, but also for pathologies such as cancers for which the amount of amino acids may be also severely affected in the cellular microenvironment.

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