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Deprivation of protein or amino acid induces C/EBP β synthesis and binding to amino acid response elements, but its action is not an absolute requirement for enhanced transcription

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Abstract

A nutrient stress signalling pathway is triggered in response to protein or amino acid deprivation, namely the AAR (amino acid response), and previous studies have shown that C/EBP β (CCAAT/enhancer-binding protein β) expression is up-regulated following activation of the AAR. DNA-binding studies, both *in vitro* and *in vivo*, have revealed increased C/EBP β association with AARE (AAR element) sequences in AAR target genes, but its role is still unresolved. The present results show that in HepG2 human hepatoma cells, the total amount of C/EBP β protein, both the activating [LAP* and LAP (liver-enriched activating protein)] and inhibitory [LIP (liver-enriched inhibitory)] isoforms, was increased in histidine-deprived cells. Immunoblotting of subcellular fractions and immunostaining revealed that most of the C/EBP β was located in the nucleus. Consistent with these observations, amino acid limitation caused an increase in C/EBP β DNA-binding activity in nuclear extracts and chromatin immunoprecipitation revealed an increase in C/EBP β binding to the AARE region *in vivo*, but at a time when transcription from the target gene was declining. A constant fraction of the basal and increased C/EBP β protein was phosphorylated on Thr²³⁵ and the phospho-C/EBP β did bind to an AARE. Induction of AARE-enhanced transcription was slightly greater in C/EBP β -deficient MEFs (mouse embryonic fibroblasts) or C/EBP β siRNA (small interfering RNA)-treated HepG2 cells compared with the corresponding control cells. Transient expression of LAP*, LAP or LIP in C/EBP β -deficient fibroblasts caused suppression of increased transcription from an AARE-driven reporter gene. Collectively, the results demonstrate that C/EBP β is not required for transcriptional activation by the AAR pathway but, when present, acts in concert with ATF3 (activating transcription factor 3) to suppress transcription during the latter stages of the response.

Keywords

activating transcription factor 4 (ATF4); asparagine synthetase; basic leucine zipper (bZIP); CCAAT/enhancer-binding protein (C/EBP); nutrient regulation; System A neutral amino acid transporter 2 (SNAT2)

Introduction

Although blood amino acid levels in mammals serve to dampen fluctuations in intracellular pools, a number of dietary and pathological conditions can result in decreased intracellular amino acid availability, which in turn can modulate a number of fundamental processes.

Under these circumstances, the amino acids are not only serving their role as metabolic or protein synthetic precursors, but also function as signal molecules that reflect the nutritional status of the entire organism. One of the many consequences of this amino acid-dependent signal transduction, termed the AAR (amino acid response) pathway, is a change in the transcription activity for specific genes (reviewed in [1]). Three amino acid-responsive genes for which some genomic characterization has been reported include *ASNS* (asparagine synthetase), *CHOP* [C/EBP (CCAAT/enhancer-binding protein) homology protein] and *SNAT2* (System A neutral amino acid transporter 2). The *ASNS* proximal promoter contains two *cis*-elements, NSRE-1 (nutrient-sensing response element-1) and NSRE-2, which together function as an AARE (AAR element) [2–4]. In contrast, the *SNAT2* AARE consists of a single sequence that is located within the first intron [5,6]. The 9 bp core sequence of the *ASNS* NSRE-1 site differs by 2 bp from the AARE within the *CHOP* and *SNAT2* genes [6,7]. ATF4 (activating transcription factor 4) protein synthesis is rapidly increased through a translational control mechanism following amino acid limitation [8,9], binds to AARE sequences and is a potent activator of AARE-containing genes [4,6,10]. Using the *ASNS* AARE as bait in a yeast one-hybrid screen to search for ATF4-binding partners, members of the C/EBP family were identified, and C/EBP β synthesis was discovered to be up-regulated following activation of the AAR pathway [11]. Binding studies, *in vitro* by EMSA (electrophoretic mobility-shift assay) and *in vivo* by ChIP (chromatin immunoprecipitation), have revealed C/EBP β binding at the *ASNS* [11,12] and *SNAT2* [6] AAREs. However, the functional role of C/EBP β during AAR-triggered transcription remains unresolved because there is evidence for both an activating [11] and a suppressing [12] action.

Based on enhanced transcription factor synthesis and ChIP analysis, Chen and co-workers proposed a working model for amino acid-regulated transcription via an AARE that represents a ‘self-limiting activation-repression programme’ that is composed of two phases [1,4,12]. Phase I encompasses the first 4 h and Phase II covers the time from 4–24 h after amino acid limitation of mammalian cells. During the first 30 min after amino acid removal from the medium, translational control of ATF4 synthesis results in increases in the following events: ATF4 protein content in the nucleus, binding of ATF4 to an AARE site, histone modification and recruitment of RNA polymerase II. In Phase II of amino acid deprivation, C/EBP β binding at the AARE increases at a time when the transcription rate has reached a peak and is beginning to decline. ATF3 synthesis and its recruitment to the AARE also increases during Phase II. Subsequent analysis of several additional AARE-containing genes has documented that the relative temporal relationships between these three factors are qualitatively similar, regardless of the target gene [4]. It is proposed that ATF3 and C/EBP β may act in concert to suppress, but not completely inhibit, the AARE-enhanced transcription [12]. However, additional data to support the proposal that C/EBP β acts as a suppressor, rather than its more common function of activation, is necessary.

The C/EBP family of transcription proteins represents a subclass of the bZIP (basic leucine zipper) family of transcription proteins [13,14]. C/EBP proteins can homodimerize, heterodimerize with other C/EBP members or heterodimerize with other bZIP members [15]. The C/EBP β mRNA is subject to alternative translational start site selection from three different methionine codons within the sequence, such that three protein isoforms are produced [16]. Human LAP* and LAP (liver-enriched activating protein) are 345 and 322 amino acids in length respectively, whereas LIP (liver-enriched inhibitory protein) represents the C-terminus of LAP*/LAP and is 147 amino acids long. As the shortest isoform, LIP lacks the transactivation domain contained within the N-terminal portion of LAP* or LAP, but retains the DNA-binding and bZIP dimerization regions and therefore acts as a naturally occurring dominant-negative repressor of C/EBP function [17]. Within the present study, the general term C/EBP β will be used to refer to the gene or the mRNA. If the experimental design can

distinguish among the isoforms, the terms LAP*, LAP and LIP will be used. C/EBP β function is critical to differentiation of adipocytes [18], neurons [19] and macrophages [20], and it has been reported to have both positive and negative influences on cell cycle control [21]. In the liver, C/EBP β regulates hepatocyte proliferation [22,23] and lipid metabolism [24] and contributes to gluconeogenesis through regulation of phosphoenolpyruvate carboxykinase [25]. At the level of individual target genes, LAP* and LAP are generally considered to be transcriptional activators, but recently Wiper-Bergeron et al. [26] reported that C/EBP β can function as a repressor of Runx2 gene transcription. Those authors documented C/EBP β binding to the Runx2 gene by ChIP.

The purpose of the present study was to extend previous work on the regulation of C/EBP β biosynthesis and to investigate its contribution to the transcriptional regulation of AARE-containing genes, by using the *ASNS* and *SNAT2* genes as model systems. The results show that in HepG2 human hepatoma cells, the total amount of C/EBP β is increased in histidine-deprived cells, the protein is largely localized to the nucleus and the increase in nuclear AARE binding activity parallels the increase in C/EBP β protein content. Following activation of the AAR pathway, transcription from the AARE-regulated gene *Asns* was actually increased in *c/ebp β ^{-/-}* MEFs (mouse embryonic fibroblasts) or in HepG2 cells transfected with an siRNA (small interfering RNA) specific for C/EBP β . Consistent with these results, recruitment of the AAR mediator ATF4 and RNA polymerase II to the *Asns* promoter still occurred in the *c/ebp β ^{-/-}* MEF cells. Over-expression of the individual C/EBP β isoforms in *c/ebp β ^{-/-}* MEF cells demonstrated that LAP* and LAP inhibited the AAR-induced transcription from an *ASNS* promoter-luciferase reporter construct, whereas LIP inhibited both the basal and the up-regulated transcription activity. Collectively, the results indicate that C/EBP β functions as a transcriptional suppressor during the amino acid-sensing programme that regulates transcription from AARE-containing genes.

Materials and Methods

Cell culture and protein diets *in vivo*

To assess the effect of protein deprivation *in vivo* on C/EBP β gene expression, Sprague–Dawley rats (Harlan, Indianapolis, IN, U.S.A.) were placed on a control diet of 19% protein (Purina) or a protein-poor diet of 8% protein (made isocaloric with the addition of sucrose) for 3 days prior to isolation of liver RNA and protein samples. The animals provided the 19% protein diet were divided into two groups, one was fed *ad libitum*, and the other was pair-fed relative to the food intake of the 8% group the day before. These studies were approved by the University of Florida Institutional Animal Care and Use Committee. Total RNA and tissue protein extracts were prepared from liver as described below. For the tissue culture studies, HepG2 human hepatoma cells were cultured in MEM (minimal essential medium; Mediatech, Herndon, VA, U.S.A.) (pH 7.4) supplemented to contain 1× non-essential amino acid solution (Mediatech), 4 mM glutamine, 25 mM NaHCO₃, 100 µg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 µg/ml amphotericin B and 10% (v/v) fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA, U.S.A.). MEFs from wild-type and *c/ebp β ^{-/-}* mice were kindly provided by Dr Peter Johnson [NIH (National Institutes of Health), Bethesda, MD, U.S.A.] [27]. MEF cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with glutamine (Mediatech), supplemented with 1× non-essential amino acids, 10% fetal bovine serum, 100 µg/ml streptomycin sulfate, 100 units/ml penicillin G and 0.25 µg/ml amphotericin B. Cells were maintained at 37 °C in a 5% CO₂/95% air incubator. For all experiments, cell cultures were replenished with fresh medium and serum for 12 h prior to initiating all treatments to ensure that the cells were in the basal ('fed') state. Amino acid deprivation was induced by transfer of cells to culture medium either lacking histidine or containing 2 mM HisOH (histidinol). HisOH blocks charging of histidine on to the corresponding tRNA and thus mimics histidine deprivation,

thereby triggering activation of the AAR cascade [28]. We document below that HisOH treatment mirrors histidine-deficient medium with regard to induction of the AAR pathway (Figure 2) and similar data have been reported previously for *ASNS* transcription [29]. For the incubation period of histidine deprivation, the medium was supplemented with 10% dialysed fetal bovine serum (Sigma, St Louis, MO, U.S.A.).

Immunoblotting and immunohistochemistry

Antibodies, obtained from Cell Signaling Technology (Danvers, MA, U.S.A.), were: a rabbit polyclonal antibody against the N-terminus of LAP^{*}/LAP that does not detect LIP (no. 3087), Thr²³⁵ P-C/EBP β (phospho-C/EBP β ; no. 3084) and Ser⁵¹ phospho-eIF2 α (eukaryotic initiation factor 2 α ; no. 9721). Antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) were: a monoclonal antibody against the C/EBP β C-terminus that detects all three isoforms (sc-7962), C/EBP α (sc-61), C/EBP δ (sc-636), C/EBP ϵ (sc-158), ATF4 (sc-200), total eIF2 α (sc-7629) and TAFII250 (sc-17134). The β -actin (A2066) antibody was from Sigma Chemical (St Louis, MO, U.S.A.). Total cell extracts were prepared by dissolving the cells directly into Laemmli sample dilution buffer (Bio-Rad, Hercules, CA, U.S.A.) and cytoplasmic or nuclear protein extracts were prepared using the NE-PER kit from Pierce Chemical Co. (Rockford, IL, U.S.A.). Extracts were isolated at the time points indicated and 30 μ g per lane was separated on a 10.5–14% Tris/HCl polyacrylamide gel (Bio-Rad) and electrotransferred to a Trans-Blot membrane (Bio-Rad). The membrane was stained with Fast Green to check for equal loading and then incubated with 5% blocking solution {5% (w/v) Carnation non-fat dry milk, in TBST [30 mM Tris base, pH 7.5, 200 mM NaCl and 0.1% (v/v) Tween 20]} for 2 h at room temperature (23 °C) with mixing. Each antibody was used at a concentration of 0.2–0.4 μ g/ml in 5% milk blocking solution and incubated for 1–2 h at room temperature or overnight at 4 °C. The blots were washed for 5 $\times$ 5 min with 1% milk blocking solution on a shaker and then incubated with the appropriate peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) for 1 h at room temperature. The blots were then washed for 5 $\times$ 5 min with freshly made TBST. The bound secondary antibody was detected using an ECL[®] kit (GE Healthcare, Piscataway, NJ, U.S.A.) and exposing the blot to a Biomax MR film (Kodak, Rochester, NY, U.S.A.).

For immunostaining, HepG2 hepatoma cells were plated on to 22 mm $\times$ 22 mm Corning glass coverslips in 6-well plates. Cells were incubated in either amino-acid-complete MEM or MEM containing 2 mM HisOH for 24 h, then washed three times with PBS and fixed in methanol for 5 min at 20°C. After fixation, cells were incubated in blocking solution containing 5% (v/v) normal goat serum in PBS overnight at 4°C. Cells were then stained with the mouse monoclonal antibody against total C/EBP β at a 1:100 dilution for 1 h at room temperature. Alexa Fluor[®] 555-conjugated goat anti-mouse IgG was used as a secondary antibody at a dilution of 1:1000 (Molecular Probes, Eugene, OR, U.S.A.). All antibodies were diluted in PBS containing 5% normal goat serum. Following the incubation in secondary antibody for 1 h at room temperature, Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, U.S.A.) was used to mount the slip and stain the nuclei.

Transient transfection assay

The cDNA for each of the C/EBP β isoforms was prepared by RT-PCR (reverse transcription-PCR) from HepG2 RNA and confirmed by sequencing after cloning into the pcDNA3.1(–) expression vector. The *ASNS* promoter-firefly luciferase plasmid was generated in the pGL3-basic plasmid (Promega, Madison, WI, U.S.A.) by placing nt –115/+51 of the human *ASNS* gene in front of the luciferase gene. MEFs (kindly supplied by Dr Peter Johnson) from *c/ebp β ^{–/–}* mice were transfected with Superfect transfection

reagent (Qiagen) at a ratio of 3:1 (Superfect/DNA) according to the manufacturer's protocol. Briefly, *c/ebpβ*^{-/-} cells were seeded on to 24-well plates (4×10^4 cells per well) and cultured for 16–18 h prior to transfection. The *ASNS*/luciferase reporter construct (1 μg) was transfected alone or with 100 ng of a *C/EBPβ* expression vector. The total amount of transfected DNA was kept constant among the experimental groups by the addition of empty pcDNA3.1 plasmid DNA. After transfection and a subsequent 24 h recovery in complete DMEM, cells were then incubated for an additional 8 h in either fresh DMEM or DMEM containing 2 mM HisOH. The cells were harvested and the effect of *C/EBPβ* overexpression was measured by determining luciferase activity using an assay system according to the manufacturer's protocol (Promega). The data are expressed as the means ± S.D. for at least four assays, and each experiment was repeated with multiple batches of cells.

Steady-state mRNA detection and transcription rate determination

Total RNA was isolated from rat liver tissue, HepG2 cells or MEF cells using the Qiagen RNeasy kit (Qiagen), including a DNase I treatment before the final elution to eliminate DNA contamination, according to the manufacturer's protocol. As published previously for the human *ASNS* gene [12], to measure the transcription activity from the mouse *ASNS* gene, an oligonucleotide primer corresponding to the mouse *ASNS* intron 4 (5'-CTTGTTTGTGGCGGTTCATTAC-3') and another from exon 5 (5'-ACGCAGATTGTTCTTCAGGGTCTC-3') were used to measure the unspliced hnRNA (heteronuclear RNA). This procedure for measuring transcription activity is based on that described by Lipson and Baserga [30], except that the hnRNA levels in the present studies were analysed by qPCR (quantitative real-time PCR). To measure the steady-state *SNAT2* and *C/EBPβ* mRNA levels in rat tissue, the primers were as follows: for *snat2* (nt + 1220 to + 1287), sense, 5'-TTTAATGTGAGCAATGCGATTGTG-3' and antisense, 5'-AGAGGAATTCCAGTATTAGC-3'; for *c/ebpβ* (within exon 4), sense, 5'-AGAACGAGCGGCTGCAGAAGA-3', and antisense, 5'-AGAGGAATTCCAGTATTAGC-3'. Reactions without reverse transcriptase were performed as a negative control to rule out amplification from any residual genomic DNA, and these tests were always negative. The reactions were incubated at 50°C for 30 min followed by 95°C for 15 min to activate the Taq polymerase and then amplification for 35 cycles at 95°C for 15 s and 60°C for 60 s. After PCR, melting curves were acquired by stepwise increase of the temperature from 55 to 95°C to ensure that a single product was amplified in the reaction.

Nuclear extract preparation and DNA binding analysis by EMSA and DAPA (DNA-affinity purification analysis)

HepG2 cells were seeded on to 150-mm dishes at a density of 15×10^6 cells per dish and the MEFs were seeded at 6.5×10^6 cells per dish. After 30 h of culture, the cells were replenished with fresh culture medium and serum for 16 h and then transferred for 6 h to medium with or without 2 mM HisOH. The nuclear extracts were prepared using the NE-PER kit from Pierce Chemical Co. The DAPA was performed using the procedures described by Deng et al. [31]. The protein concentration was determined by a modified Lowry assay using BSA as a standard [32]. For DAPA, the DNA affinity probe was an oligonucleotide (5'-CCTCGCAGGCATGATGAACTTCCCGCACGCGTTACAGGAGCCAG-3'), corresponding to nt -79/-35 and containing the AARE of the *ASNS* gene (underlined), biotinylated on both 5'-ends (synthesized by Sigma-GenoSys). For the binding reaction, 5 μg of biotinylated DNA probe and 500 μg of HepG2 nuclear extract were added to 80 μl of 4% streptavidin-agarose beads (Sigma). The final volume was adjusted to 500 μl with nuclear extract buffer (PBS containing phosphatase and protease inhibitors) and, after 1 h of incubation on a rotator at room temperature, the mixture was centrifuged at 1000 g for 1

min. The supernatant was removed and the pellet containing protein–DNA beads was washed four times with 1 ml of ice-cold PBS. After the last wash, the beads were resuspended in 100 μ l of Laemmli sample buffer (Bio-Rad) and boiled for 5 min and then aliquots of 25–50 μ l were used for immunoblot analysis.

For EMSA, single-stranded oligonucleotides covering *ASNS* nt –79 to –35 were annealed by adding 0.4 nmol of each strand with 10 μ l of 10 $\times$ annealing buffer (100 mM Tris/HCl, pH 7.5, 1 M NaCl, 10 mM EDTA) in a total volume of 100 μ l. The oligonucleotide solution was heated to 95°C for 5 min and then allowed to cool gradually to 4°C over 2 h. The double-stranded oligonucleotides were radiolabelled by extension of overlapping ends with Klenow fragment in the presence of [α -³²P]dATP. For each binding reaction, 5 μ g of nuclear extract protein was incubated for 20 min on ice with 40 mM Tris base (pH 7.5), 200 mM NaCl, 2 mM dithiothreitol, 10% (v/v) glycerol, 0.05% (v/v) Nonidet P40, 2 μ g of poly(dI-dC)·(dI-dC) (GE Healthcare), 0.4 pmol of unrelated DNA and 0.05 mM EDTA. The radiolabelled probe was added at a concentration of 0.002 pmol per reaction [$\sim$ 20000 cpm (counts per minute)], and unlabelled competitor oligonucleotides were added at the indicated concentrations. The reaction mixture, 20 μ l final volume, was incubated at room temperature for 20 min. If an antibody was tested for supershift, it was added and a second 20 min incubation was included. The reactions were subjected to electrophoresis and autoradiography as described previously [11].

ChIP analysis

Wild-type and MEF cells deficient in *c/ebp β* were seeded at 6×10^6 per 150-mm dish in complete DMEM medium and the HepG2 hepatoma cells were seeded at 15×10^6 per 150-mm dish in MEM. The cells were grown for 24 h and then transferred to fresh culture medium 12 h before transfer to control medium, medium containing 2 mM HisOH, or medium lacking histidine for the time period indicated in each Figure. ChIP analysis of the *ASNS* and *SNAT2* genes was performed as described previously [12]. The primers to amplify nt –151 to –83 of the murine *ASNS* promoter were sense, 5'-TCGGCCCCAGGATGCACGT-3' and antisense, 5'-TGGCCCGCAGTGCTGACGA-3', whereas the primers to amplify the regions of the human *SNAT2* gene have been reported previously [6]. For qPCR, serial dilutions of input chromatin were used to generate a standard curve for determining the relative amount of product. Duplicates for both the standards and the samples were simultaneously amplified using the same reaction master mixture. After PCR, melting curves were acquired by stepwise increases in the temperature from 55 to 95°C to ensure that a single product was amplified in the reaction. The results are expressed as the ratio to a 1:20 dilution of input DNA. Samples from at least three independent immunoprecipitations were analysed in duplicate and the means $\pm$ S.E.M. between conditions were compared by the Student's *t* test.

siRNA transfection

The *C/EBP β* siRNA (no. D-006423-01), siControl Non-targeting siRNA (no. D-001210-02) and DharmaFect-4 transfection reagent were purchased from Dharmacon (Lafayette, CO, U.S.A.). HepG2 cells were seeded on to 12-well plates at a density of 2.5×10^5 cells per well in MEM and grown for 16 h. Transfection was performed according to Dharmacon's instructions using 3 μ l of DharmaFect-4 and 80 nM per well final siRNA concentration. HepG2 cells were treated with transfection reagent for 24 h, then rinsed with PBS, given fresh MEM, and cultured for another 24 h. Medium was then removed and replaced with control MEM or MEM containing 2 mM HisOH. RNA or protein was isolated at specific times and analysed by RT-PCR or immunoblotting respectively.

Results

Induction of hepatic C/EBP β expression *in vivo* by a protein-poor diet

Rats were placed on a control diet of 19% protein or a protein-poor diet of 8% protein for 3 days. To monitor activation of the AAR pathway, the ATF4 protein content and the mRNA for an AAR target gene were measured in three animals for each dietary condition (Figure 1). The ATF4 level was at or below detection in response to the 19% diet, either pair-fed or fed *ad libitum*, whereas ATF4 was clearly elevated in the low-protein diet, documenting activation of the AAR. Consistent with this ATF4 expression, the mRNA for the SNAT2 amino acid transporter, a known AAR target gene [5,6], was elevated by approx. 5-fold compared with the pair-fed control (Figure 1B). Measurement of the C/EBP β mRNA documented an increase of greater than 2-fold in response to the low-protein diet (Figure 1C), consistent with previous reports [33,34]. To investigate the mechanism of action of C/EBP β in the AAR pathway at the molecular level, additional experiments were performed *in vitro* using HepG2 human hepatoma cells and c/ebp β -deficient MEFs.

C/EBP β expression induced by amino acid limitation in cultured cells

Amino acid deprivation was induced by transfer of cells to culture medium either lacking histidine or containing 2 mM HisOH. HisOH blocks charging of histidine on to the corresponding tRNA and thus mimics histidine deprivation, thereby triggering activation of the AAR cascade [28]. We have previously documented that HisOH treatment mirrors histidine-deficient medium with regard to induction of *ASNS* transcription [29], and Figure 2 further illustrates that the AAR pathway, as monitored by phosphorylation of eIF2 α , is activated equally by either treatment. That the response to the combination of no histidine and the presence of HisOH is not additive provides additional support for a common mechanism.

Immunoblotting with a polyclonal antibody against the C/EBP β N-terminal region, which recognizes the LAP* and LAP isoforms, indicated that both were induced in amino-acid-deprived HepG2 human hepatoma cells (Figure 3). A time course revealed that the elevated LAP*, LAP and LIP total protein content tended towards an enhanced amount after 4 h, but was maximal at the 12 h time point, at which the densitometry estimates showed an approx. 3–4-fold increase. These results are consistent with previously published ChIP analysis documenting increased C/EBP β binding to the *ASNS* promoter, which was maximal at 8–12 h after amino acid removal from the medium [12].

Nuclear localization of C/EBP β

The functional activity of C/EBP β has been documented in other circumstances to be regulated, in part, by nuclear translocation and subnuclear localization [35–37]. To monitor the expression and subcellular localization of C/EBP β during amino acid limitation, both immunostaining and immunoblotting of cell fractions were employed. When HepG2 hepatoma cells were incubated in MEM or MEM containing HisOH for 4 h and then immunostained with a C/EBP β antibody that recognizes all three isoforms, most of the C/EBP β protein in HepG2 cells was localized to the nucleus (Figure 4A). Activation of the AAR pathway caused a detectable increase in staining intensity in most, but not all, cells. It was noted that a small percentage of cells in the control medium stained as intensely as those incubated in HisOH and, vice versa, not all of the cells maintained in the HisOH-containing medium exhibited the same degree of brightness (Figure 4A). Negative controls documented that there was no staining in cultured MEFs from C/EBP β ^{-/-} mice and that there was no immunostaining of HepG2 cells if the incubation lacked primary antibody (results not shown). Consistent with the immunostaining, when isolated cytoplasmic and nuclear protein fractions were subjected to immunoblotting, most of the C/EBP β protein was

present in the nuclear fraction and an increase in the abundance was observed following amino acid limitation (Figure 4B).

Binding of C/EBP β to an AARE

Previous EMSA and ChIP analysis has documented that C/EBP β can bind to either the *ASNS* [11] or the *SNAT2* [6] AARE sequence. However, without isoform-specific antibodies, neither EMSA bandshift nor ChIP analyses can document which isoform is bound. To distinguish between the isoforms, DAPA was performed [31]. The principle of this *in vitro* binding assay is similar to an EMSA, but the oligonucleotide probe is biotinylated and pulled down with streptavidin beads. This pull-down technique allows for the subsequent analysis of the bound proteins by immunoblotting. Therefore the DAPA approach is useful in conjunction with antibodies that are not concentrated enough to perform traditional EMSA bandshifts or for DNA-binding proteins that have multiple isoforms, like C/EBP β . In the present studies, nuclear extracts were isolated from HepG2 cells incubated in MEM or MEM containing HisOH and then tested for binding activity using a double-stranded DNA oligonucleotide containing the *ASNS* AARE as a probe followed by immunoblotting for C/EBP β (Figure 5). A basal amount of C/EBP β protein was present in the nuclear extract from control cells ($t=0$) and, consistent with the immunoblotting results, beginning at the 4 h point of HisOH treatment, the amount of LAP* and LAP bound to the AARE increased. LIP binding activity was detectable, but the amount was not substantially changed following HisOH treatment (Figure 5). As a positive control, ATF4 binding was monitored and the results agreed with previously published EMSA [10] and ChIP [12] analyses documenting a high degree of induced ATF4 binding within 1 h after amino acid limitation.

Phosphorylation of C/EBP β by the MAPK (mitogen-activated protein kinase) pathway

Amino acid limitation activates the MAPK, as judged by MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase]-mediated phosphorylation of ERK [38–40]. Activation of the MEK/ERK signal transduction pathway by a variety of mechanisms leads to increased phosphorylation of human LAP at Thr²³⁵ [18,19,36,41], but the effect of amino acid deprivation on Thr²³⁵ phosphorylation has not been reported. Franchi-Gazzola et al. [38] showed that the MEK inhibitor PD98059 blocked the induction of System A transport activity after amino acid limitation of human fibroblasts. To test the effect of amino acid availability on C/EBP β phosphorylation, HepG2 cells were incubated in MEM or MEM containing HisOH for 6, 8 and 12 h in the presence or absence of the MEK inhibitor PD98059 and then protein extracts were immunoblotted for either total C/EBP β or Thr²³⁵ P-C/EBP β (Figure 6). Consistent with the data in Figure 3, the total amount of LAP increased (Figure 6A). The amount of Thr²³⁵ P-C/EBP β was nearly undetectable in the basal state, but there was a substantial increase in the amount of phosphorylation in response to HisOH treatment (Figure 6B). When the ratio of Thr²³⁵ P-C/EBP β to the total was calculated at each time point, the value remained unchanged, indicating that the amount of protein undergoing phosphorylation was proportional to the total. Therefore amino acid limitation does not preferentially enhance C/EBP β phosphorylation at Thr²³⁵, but as the total amount of C/EBP β is increased, the relative amount of P-C/EBP β is also increased. Interestingly, the specificity of inhibition of MEK on C/EBP β phosphorylation could not be elucidated because the increase in total C/EBP β protein was also blocked by PD98059 treatment of the cells (Figure 6A). These data indicate that the induction of C/EBP β synthesis during the AAR is dependent on MEK activity and the basis for that observation will be published elsewhere (M.M. Thiaville, Y.-X. Pan, A. Gjymishka, C. Zhong and M.S. Kilberg, unpublished work).

To determine whether Thr²³⁵ P-C/EBP β binding to an AARE occurred *in vivo*, ChIP analysis was performed in HepG2 cells incubated in complete MEM or MEM lacking histidine (Figure 6C). To investigate the specificity of P-C/EBP β binding, primers were prepared to amplify distinct regions of the *SNAT2* transporter gene because the AARE in *SNAT2* is located in the first intron, 700 bp downstream of the proximal promoter, and therefore binding to the promoter versus the AARE region can be distinguished [5]. Binding of total C/EBP β to the AARE region is enhanced following amino acid limitation [42]. ChIP analysis documented that association of Thr²³⁵ P-C/EBP β with the promoter and protein-coding regions was relatively low and there was no significant enhancement by histidine deprivation (Figure 6C). The level of Thr²³⁵ P-C/EBP β binding to the intronic AARE region in cells maintained in amino-acid-complete MEM was relatively small, but greater than the background amount observed for a non-specific IgG. However, in response to amino acid limitation, the Thr²³⁵ P-C/EBP β binding was enhanced by more than 4-fold (Figure 6C), a value similar in magnitude to the increase in total C/EBP β binding previously reported [12].

Transcription from an AARE-regulated gene in C/EBP β -deficient cells

To determine whether C/EBP β is absolutely essential for amino acid-dependent regulation of transcription from an AARE-containing gene, *c/ebp β ^{+/+}* or *c/ebp β ^{-/-}* MEF cells were incubated with or without HisOH for 2, 4 or 8 h prior to isolation of total RNA. The transcription activity of the *Asns* gene was measured by analysing the synthesis of hnRNA [12]. Activation of the AAR pathway resulted in increased *Asns* transcription in both cell populations, but the magnitude of the response was different in that the maximum activity in the *c/ebp β ^{-/-}* cells was greater than that in the *c/ebp β ^{+/+}* cells (Figure 7A). For both cell populations, the induction was transient, as reported previously for hepatoma cells [12], such that beyond 8 h the values were nearly the same (Figure 7A). Given that the amino-acid-dependent synthesis of ATF4 has not been investigated in *c/ebp β ^{-/-}* cells, as a positive control, cell extracts were immunoblotted to document increased ATF4 content (Figure 7B).

One of the concerns in using MEF cells prepared from gene-knockout mice is that they have been selected to grow in culture in the absence of the deleted gene. To demonstrate C/EBP β -independent activation of AARE-driven transcription in a cell type not subjected to such a selection, HepG2 cells were transfected with siRNA against C/EBP β and then assayed for *ASNS* transcription following 2 or 8 h of HisOH treatment (Figure 7C). The siRNA was effective in reducing the level of both C/EBP β mRNA and protein expression (Figure 7D). The basal transcription activity measured in control MEM was not affected by knockdown of C/EBP β , but after activation of the AAR pathway there was an increase in transcription activity in both cell populations, and in the cells with reduced C/EBP β there was a trend towards a higher level at 2 h that reached statistical significance at 8 h (Figure 7C).

Antagonism of amino acid deprivation by overexpression of C/EBP β isoforms in *c/ebp β -deficient MEF cells*

To determine the effect of the three C/EBP β isoforms on AARE-driven transcription independently of one another, each was transiently expressed in *c/ebp β ^{-/-}* MEF cells and transcription from an *ASNS* promoter-luciferase reporter construct was measured after HisOH treatment (Figure 8). Both LAP* and LAP had little or no effect on the basal level of *ASNS* promoter-driven expression, but completely blocked the induction following HisOH treatment (Figure 8). In contrast, the LIP isoform, recognized as a naturally occurring dominant negative for C/EBP β action, strongly inhibited both basal and activated transcription.

Composition of AARE complexes in C/EBP β -deficient cells

To investigate the possibility that one of the other C/EBP proteins might functionally replace C/EBP β in the deficient MEF cells, nuclear extracts from *c/ebp β ^{+/+}* (labelled WT in Figure 9) and *c/ebp β ^{-/-}* (labelled KO in Figure 9) MEF cells were subjected to EMSA using an AARE-containing oligonucleotide probe. In the wild-type cells, there were two primary complexes formed (labelled C1 and C2 in Figure 9) and activation of the AAR pathway caused the abundance of both to increase, although the amount of C1 increased to a greater degree than C2 (lane 1). In the presence of C/EBP β antibody there were three SCs (shifted complexes) [SC1 (shifted complex 1), SC2 and SC3] formed using WT extracts with a noticeable loss of C1 in the extracts from HisOH-treated cells (lane 5). The abundance of these three SCs was also detectable in the WT extracts incubated with C/EBP δ antibody, especially SC2 (lane 7), but much less so with antibodies against C/EBP α (lane 3) or C/EBP ϵ (lane 9). In the *c/ebp β ^{-/-}* cells incubated in the control MEM, the amount of C2 was much greater than C1 and neither was significantly shifted by antibody against C/EBP α , C/EBP β , C/EBP δ or C/EBP ϵ (lanes 4, 6, 8 and 10). When the *c/ebp β ^{-/-}* cells were subjected to HisOH, the increase in abundance of both C1 and C2 was again evident (lane 2). However, as expected for MEF cells lacking C/EBP β expression, inclusion of the C/EBP β antibody did not cause a supershift of the induced complexes (lane 6); an observation in contrast with the supershift by anti-C/EBP β in the wild-type cells (lane 5). Nor did the C/EBP δ antibody cause a supershift of the induced complexes in the C/EBP β -deficient cells (lane 8), suggesting that C/EBP δ binding requires C/EBP β . These results indicate that although C/EBP β , and to a lesser extent C/EBP δ , are present in the AARE-bound complexes after amino acid limitation of MEF cells, their presence is not an absolute requirement for these complexes to form. To determine the role of C/EBP β in the formation of ATF4-containing AARE complexes, the binding incubations were performed in the presence of ATF4 antibody (lanes 11 and 12). ATF4 antibody caused the loss of both C1 and C2 in WT extracts from both MEM- and HisOH-treated cells and led to an increase in the amount of SC3 and to a lesser extent SC2 (lane 11). Using the extracts from the *c/ebp β ^{-/-}* MEF cells did not alter the migration of the ATF4 supershifts and actually enhanced the amount of SC3 (compare lanes 11 and 12). These results suggest that despite the fact that all of the known AARE sequences are considered C/EBP-ATF composite sites [1,43,44], the ATF4-containing complexes bound at the AARE following activation of the AAR pathway do not absolutely require a C/EBP-binding partner.

Previous studies in HepG2 hepatoma cells have revealed a self-limiting temporal programme of histone modifications and sequential recruitment of a number of bZIP transcription factors that appears to be qualitatively conserved among many AARE-containing genes [4,12]. Those studies showed that after 8 h of amino acid limitation in HepG2 cells, ATF4, ATF3 and C/EBP β are all bound to the AARE. Therefore wild-type and *c/ebp β ^{-/-}* MEF cells were subjected to ChIP analysis following HisOH treatment for 8 h (Figure 10). As expected from previous studies in HepG2 hepatoma cells [12], after amino acid limitation of the wild-type cells, ChIP analysis of the *ASNS* gene showed that the acetylation of histone H3, recruitment of RNA polymerase II, and binding of ATF4, ATF3, and C/EBP β were increased. In the wild-type cells, the binding of C/EBP α and C/EBP δ was not enhanced significantly by histidine deprivation. Given the lack of C/EBP β expression in the *c/ebp β ^{-/-}* MEF cells, the binding values obtained by ChIP assay using the C/EBP β antibody can be taken as a non-specific background (Figure 10). Relative to the C/EBP β values, there also was not a significant amount of AARE binding by either C/EBP α or C/EBP δ . These negative results are consistent with the EMSA data showing that neither of these bZIP proteins replaces or compensates for the loss of C/EBP β . Interestingly, despite the lack of C/EBP β in these cells, ChIP analysis of the knockout cells documented that the expected increases occurred in chromatin modification, as judged by histone H3 acetylation,

ATF4 and ATF3 binding, and recruitment of RNA polymerase II (Figure 10). These results support the data showing activation of transcription in the absence of C/EBP β (Figure 7A) and the ability to form ATF4-containing AARE complexes in the absence of C/EBP β (Figure 9).

Discussion

Previous observations from our laboratory have demonstrated that amino acid deprivation of mammalian cells leads to increased transcription from the C/EBP β gene, synthesis of total C/EBP β protein and binding to the AARE within the *ASNS* promoter [12]. The results presented in the present study confirm the increased expression of hepatic C/EBP β in rats maintained on a protein-deficient diet and extend previous observations *in vitro* by documenting the following. (i) In human HepG2 cells, the total amount of C/EBP β protein, LAP*, LAP and LIP isoforms, is increased in histidine-deprived cells and is largely localized to the nucleus. (ii) The novel use of a DNA-affinity purification approach, rather than EMSA, permitted us to distinguish between LAP*, LAP and LIP binding. After AAR pathway activation, the increase in nuclear LAP* and LAP binding activity for an AARE sequence parallels the increase in total protein, whereas LIP binding did not increase significantly. (iii) There is an increase in phosphorylation of C/EBP β at the MAPK site of LAP Thr²³⁵, but the increase is simply proportional to the increase in total protein content. However, ChIP analysis reveals that the P-C/EBP β does indeed bind to an AARE. (iv) The induced transcription from the AARE-regulated gene *Asns* is actually enhanced in *c/ebp β ^{-/-}* MEF cells compared with wild-type cells and the same enhancement of the transcriptional response occurs when C/EBP β content is suppressed by siRNA treatment of HepG2 cells. (v) Overexpression of the individual isoforms in *c/ebp β ^{-/-}* MEF cells demonstrates that both LAP* and LAP suppress the AAR-associated induction of an AARE-containing gene, whereas LIP inhibits both the basal and the up-regulated transcriptional activity. (vi) ChIP analysis of the *c/ebp β* -deficient MEF cells documents that after AAR activation, the chromatin modification and the enhanced recruitment of the transcriptional activator ATF4, RNA polymerase II and the transcriptional repressor ATF3 all occurred as expected. These results demonstrate that activation of a gene via an AARE site and recruitment of other critical transcription factors are not dependent on C/EBP β .

Marten et al. [45] reported that the rat liver LAP content was unchanged and the LIP content was increased in response to reduced dietary protein. Those authors also showed that the DNA-binding activity for total C/EBP β , as measured by EMSA, was increased by a low-protein diet, but the lack of isoform-specific antibodies prevented them from distinguishing which isoform was bound. Guo and Cavener [34] recently reported that rats fed a leucine-deficient diet exhibited an increase in hepatic C/EBP β mRNA. The present studies confirm the increase in expression of hepatic C/EBP β mRNA in protein-deprived rats and document, in parallel, that the content of ATF4 protein, the transcriptional mediator of the AAR pathway, is enhanced as well. Marten et al. [33] also showed increased C/EBP β mRNA content in rat hepatoma cells following incubation in amino-acid-limiting medium, consistent with our results in human hepatoma cells [11,42]. Previously, we reported that overexpression of the LAP isoform increased transcription from an AARE-driven firefly luciferase reporter plasmid [11]. Those firefly luciferase activities were normalized to CMV (cytomegalovirus)-driven *Renilla* luciferase activity, as is commonly done. However, in reassessing those data, the LAP effect was the consequence of a LAP-induced decrease in *Renilla* expression because the CMV promoter and/or the pGL3 plasmid backbone contains cryptic sequences that mediate an inhibition of transcription in response to LAP. Further analysis of commercially available luciferase vectors revealed that overexpression of C/EBP β also has effects on the thymidine kinase and SV40 (simian virus 40) promoters, all of which are routinely used as 'control' promoters. Consequently, our more recent studies have

normalized the firefly luciferase activities to the protein content in each well using multiple wells per experiment and multiple experiments with independent cell batches to statistically eliminate the possibility of transfection artefact. Using this approach, Chen et al. [12] documented that when C/EBP β is co-expressed with ATF3 in HepG2 cells, it acts in concert with ATF3 to suppress the ATF4-dependent activation of an AARE-containing gene. The present overexpression results extend those observations in *c/ebp β* -deficient MEF cells, to eliminate the confounding variable of endogenous C/EBP β expression, as in the HepG2 cells. Those *c/ebp β* ^{-/-} data show that the LAP* and LAP isoforms function to suppress AARE-driven promoter activity in response to amino acid limitation. The conclusion that all three isoforms exhibit a suppressive effect is consistent with the timing of the increased *de novo* synthesis of LAP*, LAP and LIP shown here, which occurs during a period of the AAR when transcription from AARE-containing genes has reached a peak and is beginning to slow [4]. Furthermore, the increased transcription activity, observed after employing the two completely independent C/EBP β knockdown approaches of *c/ebp β* ^{-/-} MEF cells and HepG2 cells treated with C/EBP β siRNA, is consistent with the conclusion that C/EBP β functions as a transcriptional suppressor at AARE sites.

It has been documented by several laboratories that C/EBP β can be phosphorylated by ERK on Thr²³⁵ (residue number for LAP) in response to activation of the MAPK pathway [41,46]. Consistent with the known activation of ERK by amino acid limitation [38,39], the present results show that C/EBP β is phosphorylated at Thr²³⁵ following HisOH treatment, but the magnitude of the increase in Thr²³⁵ P-C/EBP β is simply proportional to the increase in total protein. This result suggests that amino acid limitation does not cause a preferential enhancement of phosphorylation. However, the ChIP analysis showed that the P-C/EBP β does bind to the SNAT2 AARE. Future development of isoform-specific detection mechanisms as well as additional phospho-specific reagents will be necessary to gain further insight. For example, it was initially shown that ERK-mediated phosphorylation of C/EBP β at Thr²³⁵ (Thr¹⁸⁸ in the mouse) was necessary for adipogenesis, but it was later discovered that the MAPK phosphorylation at Thr²³⁵ was merely a 'priming' event for phosphorylation at additional sites by GSK3 β (glycogen synthase kinase 3 β) [18].

The ChIP data from the *c/ebp β* ^{-/-} cells indicate that neither C/EBP α nor C/EBP δ occupies the AARE in the absence of C/EBP β . Therefore the observation that activation of an AARE-containing gene does not require C/EBP β is not the result of an overlap or redundancy of function with either of these other C/EBP members. The proximal promoter region of the human *CHOP* gene contains a sequence (5'-TGATGCAAT-3') that is identical with the AARE sequence within *SNAT2* and differs from the *ASNS* AARE sequence by only 2 nt. The CHOP element was termed a 'C/EBP-ATF composite site' because it is made up of a half site for these two bZIP transcription factor subfamilies [43,44]. There was already published evidence for C/EBP β binding to this site [47], when Fawcett et al. [44] reported a transient ATF4 binding to this sequence in response to arsenite-induced stress. It was proposed by those authors that the ATF4 is replaced subsequently by ATF3, triggering a suppression of the gene towards the basal expression rate. Given the C/EBP β binding at this site, those authors also proposed that ATF4 and/or ATF3 might form heterodimeric complexes with C/EBP β . This same C/EBP-ATF composite site was later shown to function as an AARE that mediates the activation of the *CHOP* gene in response to amino acid limitation [48]. However, the present EMSA analysis using nuclear extracts from the *c/ebp β* ^{+/+} and *c/ebp β* ^{-/-} MEF cells suggests that ATF4 and C/EBP β are not necessarily components of the same complex, even though both are bound to the AARE sequence. Consistent with this interpretation, the ChIP results show the acetylation of histone H3, binding of ATF4 and the recruitment of RNA polymerase II in the *c/ebp β* ^{-/-} cells. Thus the data reported here provide evidence that C/EBP β is not an absolute requirement for the ATF4-dependent activation of transcription via an AARE. Furthermore, the subsequent

recruitment of the transcriptional repressor ATF3 in the absence of C/EBP α , C/EBP β or C/EBP δ demonstrates that formation of a C/EBP–ATF heterodimer is not an absolute requirement for the ATF3-dependent repression phase of AARE-regulated transcription. On the other hand, in wild-type cells C/EBP β is constitutively bound to the AARE in the fed state and the level increases during nutrient stress [12]. Consequently, future studies investigating post-transcriptional processing of C/EBP β and the relationship between C/EBP β , ATF3 and ATF4 action in regulating amino-acid-responsive genes will be important to advance our understanding of the integration of bZIP transcription factors in the AAR pathway.

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Abbreviations used

AAR	amino acid response
AARE	AAR element
ASNS	asparagine synthetase
ATF	activating transcription factor
bZIP	basic leucine zipper
C/EBP	CCAAT/enhancer-binding protein
ChIP	chromatin immunoprecipitation
CHOP	C/EBP homology protein
CMV	cytomegalovirus
DAPA	DNA-affinity purification analysis
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
eIF2α	eukaryotic initiation factor 2 α
EMSA	electrophoretic mobility-shift assay
ERK	extracellular-signal-regulated kinase
HisOH	histidinol
LAP	liver-enriched activating protein
LIP	liver-enriched inhibitory protein
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblasts
MEK	MAPK/ERK kinase
MEM	minimal essential medium
NSRE-1	nutrient-sensing response element-1
P-C/EBPβ	phospho-C/EBP β
qPCR	quantitative real-time PCR
RT-PCR	reverse transcription-PCR
SC1	shifted complex 1
siRNA	small interfering RNA
SNAT2	System A neutral amino acid transporter 2

Figure 1. Hepatic C/EBP β mRNA expression is increased by a protein-poor diet

Rats were placed on a control diet of 19% protein or a protein-poor diet of 8% for 3 days prior to isolation of liver RNA and protein samples. The animals provided the 19% protein diet were divided into two groups, one was fed *ad libitum* and the other was pair-fed relative to the food intake of the 8% group the day before. To monitor activation of the AAR pathway, the ATF4 protein content was analysed by immunoblotting tissue extracts (**A**), and the mRNAs for either SNAT2 transporter (**B**) or C/EBP β (**C**) were measured by qPCR using tissue from three animals for each dietary condition. The mRNA data are plotted relative to the value obtained for the 19% *ad libitum* fed animals and values that are significantly different from this control group are marked with an asterisk ($P < 0.05$).

Figure 2. The induction of the AAR pathway is enhanced by either histidine deprivation or HisOH treatment

HepG2 human hepatoma cells were incubated for 2, 4 or 8 h in complete MEM, MEM lacking histidine (MEM-His), complete MEM containing 2 mM HisOH, (MEM+HisOH), or both conditions simultaneously (MEM-His+HisOH). Whole cell extracts were subjected to immunoblotting for total eIF2 α or Ser⁵¹ phospho-eIF2 α (P-eIF2 α).

Figure 3. Time course of C/EBP β protein content in nuclear extracts from HisOH-treated cells HepG2 human hepatoma cells were incubated in MEM $\pm$ 2 mM HisOH for the time indicated. Nuclear extracts were prepared and subjected to immunoblot analysis as described in the Materials and methods section. The blots were reprobed for TAFII250 as a loading control. The blots were analysed by densitometry, the C/EBP β data were normalized to those for TAFII250 and the resulting quantification was presented as the fold induction relative to the MEM value at each time point. The results from a single blot are shown, but the results are representative of at least two independent experiments.

Figure 4. C/EBP β localizes in the nucleus of HepG2 human hepatoma cells

For (A), HepG2 cells were incubated in MEM $\pm$ 2 mM HisOH for 24 h and then immunostained for C/EBP β . Where noted, the cell nuclei were stained with DAPI. For (B), parallel dishes of cells were treated as described for (A) and then fractionated into cytoplasmic and nuclear protein fractions prior to immunoblotting for C/EBP β . The results from a single blot are shown, but the results are representative of at least two independent experiments.

Figure 5. C/EBP β DNA binding activity is enhanced by amino acid limitation

HepG2 human hepatoma cells were incubated in MEM $\pm$ 2 mM HisOH for the time indicated. Nuclear extracts were prepared and subjected to DAPA prior to immunoblotting for bound C/EBP β . As described in the Materials and methods section, the oligonucleotide probe contained the AARE sequence from the human *ASNS* gene. The resulting blots were first probed with an antibody for C/EBP β and then, as a positive control, reprobed with an antibody against ATF4.

Figure 6. After amino acid limitation, C/EBP β is phosphorylated at a MAPK site and binds to an AARE

HepG2 cells were incubated in MEM or MEM lacking histidine (MEM–His) for the time indicated. A parallel set of treated cells was incubated in the presence of 50 μ M PD98059 to inhibit MEK activation. Protein extracts were prepared and subjected to immunoblot analysis for both total C/EBP β protein (A) and C/EBP β phosphorylated at Thr²³⁵, a MAPK site (B). The blots were reprobed for β -actin as a loading control. The results from a single blot are shown, but it is representative of multiple independent experiments. For the data of (C), HepG2 cells were treated with either complete MEM or MEM lacking histidine (MEM–His) for 8 h before performing ChIP assays. PCR products amplifying the indicated regions of the *SNAT2* gene were used to perform qPCR. A rabbit anti-chicken IgG was used as the non-specific negative control (n/s IgG). Data were plotted as the ratio to the value obtained with the 1:20 dilution of input DNA.

Figure 7. The transcription activity from an amino acid regulated gene is enhanced in *c/ebpβ*-deficient cells

(A) MEF cells from *c/ebpβ*^{-/-} mice were incubated with or without 2 mM HisOH for the time indicated and then the transcription activity from the *ASNS* gene was measured. The results are plotted relative to the DMEM control value at each time point. (B) As a positive control to illustrate the activation of the AAR pathway in the *c/ebpβ*^{-/-} MEF cells, a protein extract was immunoblotted for ATF4 content. (C) HepG2 cells were transfected with an siRNA against C/EBPβ or a control siRNA, as described in the Materials and methods section. After 48 h, the cells were incubated in MEM ± 2 mM HisOH for 2 or 8 h and the transcriptional activity of the *ASNS* gene was monitored. The difference between the control siRNA and the C/EBPβ siRNA-treated cells was statistically significant at 8 h ($P < 0.05$), as designated by the asterisk. (D) An siRNA oligonucleotide corresponding to the C/EBPβ mRNA was transiently transfected into HepG2 cells. After 48 h, the cells were incubated in MEM or MEM+2 mM HisOH for 2 or 8 h and then processed for analysis of C/EBPβ protein.

Figure 8. Expression of individual C/EBP β isoforms in *c/ebp β* -deficient MEF cells
c/ebp β ^{-/-} MEF cells were transfected with expression vectors encoding LAP*, LAP or LIP. The cells were co-transfected with a reporter plasmid containing firefly luciferase driven by the AARE-containing *ASNS* promoter. The cells were then incubated in DMEM $\pm$ 2 mM HisOH for 8h prior to preparing extracts for analysis of luciferase expression, which is reported as *ASNS* promoter activity. The experimental conditions marked with an asterisk are significantly different ($P < 0.05$) from the corresponding control value (either vector/DMEM or vector/DMEM + HisOH).

Figure 9. *In vitro* binding of C/EBP proteins and ATF4 to an AARE

Prior to nuclear extract isolation, HepG2 cells were incubated for 8 h in either complete MEM or MEM +2 mM HisOH. EMSA analysis to monitor transcription factor binding to the *ASNS* AARE was performed as described in the Materials and methods section. The arrows marked C1 and C2 denote the primary complexes present in the basal state that were increased in amount when extracts from HisOH-treated cells were tested. The complexes SC1–3 were the ‘supershift’ complexes that were generated in response to incubation with antibody for the indicated protein. The autoradiographic film shown is representative of several separate experiments using independently prepared nuclear extracts.

Figure 10. ChIP analysis of the ASNS AARE region in wild-type and *c/ebpβ*-deficient MEF cells Either wild-type or *c/ebpβ*^{-/-} MEF cells were incubated in DMEM or DMEM ± 2 mM HisOH for 8 h and then protein binding associated with the *ASNS* AARE region was monitored by ChIP assays. PCR products amplifying the AARE-containing promoter of the *ASNS* gene were assayed by qPCR and the data are plotted as the ratio to the value obtained with the 1:20 dilution of input DNA.