Amino-acid limitation induces transcription from the human C/EBPβ gene via an enhancer activity located downstream of the protein coding sequence

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For animals, dietary protein is critical for the nutrition of the organism and, at the cellular level, protein nutrition translates into amino acid availability. Amino acid deprivation triggers the AAR (amino acid response) pathway, which causes enhanced transcription from specific target genes. The present results show that C/EBPβ (CCAAT/enhancer-binding protein β) mRNA and protein content were increased following the deprivation of HepG2 human hepatoma cells of a single amino acid. Although there was a modest increase in mRNA half-life following histidine limitation, the primary mechanism for the elevated steady-state mRNA was increased transcription. Transient transfection documented that C/EBPβ genomic fragments containing the 8451 bp 5′ upstream of the transcription start site did not contain amino-acid-responsive elements. However, deletion analysis of the genomic region located 3′ downstream of the protein coding sequence revealed that a 93 bp fragment contained an amino-acid-responsive activity that functioned as an enhancer. Exogenous expression of ATF4 (activating transcription factor 4), known to activate other genes through amino acid response elements, caused increased transcription from reporter constructs containing the C/EBPβ enhancer in cells maintained in complete amino acid medium. Chromatin immunoprecipitation demonstrated that RNA polymerase II is bound at the C/EBPβ promoter and at the 93 bp regulatory region in vivo, whereas ATF4 binds to the enhancer region only. Immediately following amino acid removal, the kinetics of binding for ATF4, ATF3, and C/EBPβ itself to the 93 bp regulatory region were similar to those observed for the amino-acid-responsive asparagine synthetase gene. Collectively, the findings show that expression of C/EBPβ, which contributes to the regulation of amino-acid-responsive genes, is itself controlled by amino acid availability through transcription.

Key words: activating transcription factor 3 (ATF3), activating transcription factor 4 (ATF4), amino acid response element (AARE), basic leucine-zipper (bZIP), CCAAT/enhancer-binding protein (C/EBP), nutrient starvation.

INTRODUCTION

Dietary protein intake and a balanced amino acid supply is an important factor in the general nutrition of animals, especially during development [1,2] and may have an impact on lifespan as well [3]. At the level of individual tissues and cells, protein nutrition is represented by amino acid availability. Although the amino acid content in the bloodstream and protein degradation both provide some buffering capacity for variations in dietary protein/amino acid intake, fluctuations in the intracellular levels of individual amino acids may occur in response to diet, disease and metabolic need [4]. Indeed, animals possess an innate mechanism to detect and reject a diet with an imbalanced amino acid composition [5]. Obviously, the metabolic stage of the animal must be adapted to these changes, both at the level of interorgan metabolite fluxes and at the cellular level to change the flux through individual metabolic pathways. In this context, amino acids serve as signal-transduction messengers to transmit the nutritional status of the organism to individual cells. The signalling pathway that is triggered in response to amino-acid-deprivation is referred to as the ‘amino acid response’ (AAR).

One of the mechanisms by which cells respond to amino acid stress is by increasing transcription from specific genes that are targets of the AAR pathway. These genes contain AAREs (amino acid response elements) that mediate the enhancement of transcription [6–9]. The AARE sequences function as enhancer elements [10,11] and have a 9–10 bp core that can differ in sequence by one or two nucleotides between genes. Several of the enhancer binding proteins that assemble on AAREs have been identified, including ATF2 (activating transcription factor 2) [12], ATF4 [12,13], ATF3 [14,15], and C/EBPβ (CCAAT/enhancer-binding protein β) [16]. For the amino-acid-dependent control of the ASNS (asparagine synthetase gene), C/EBPβ has been documented to be a required component [16]. Increased C/EBPβ expression itself may represent a critical AAR pathway step just prior to AARE-containing target genes [17]. Consequently, the goal of the present studies was to investigate the mechanism by which the AAR pathway leads to increased C/EBPβ expression.

C/EBPβ is a member of a family of transcription factors that also includes C/EBPa, C/EBPγ, C/EBPδ, and CHOP (C/EBP homology protein) (reviewed in [18,19]). The C/EBPβ members dimerize with other bZIP (basic leucine-zipper) family members, as well as other transcription factors [20]. C/EBPβ plays a role in a wide range of important cellular processes, such as adipocyte differentiation, carbohydrate metabolism, inflammation and cellular proliferation [18,19,21]. The C/EBPβ mRNA is subject to differential translational start site selection from each of three methionine codons within the sequence, such that three protein isoforms are produced [22]. Although C/EBPβ post-translational modification and function has been studied
Extensively, investigation of the transcriptional control of the C/EBPβ gene itself is limited. A recent report from this laboratory, documenting the presence of an unfolded protein response element [23], is the only published information on genomic element identification for the human C/EBP gene. The rat C/EBPβ promoter has been studied, and Niehof et al. [24] have demonstrated that there are two CRE (cAMP-response element)-like sequences within the rat promoter that are necessary for maintaining basal transcription. Maren et al. reported that the rat liver mRNA content for C/ebpβ was increased in response to reduced dietary protein [25] and in rat hepatoma cells following incubation in amino-acid-limiting medium [26]. Subsequently, Siu et al. [16] showed that not only is C/EBPβ mRNA increased by histidine deprivation of HepG2 human hepatoma cells, but there is also increased C/EBPβ DNA binding activity in the nuclear extract of amino-acid-deprived HepG2 cells. More recently, Chen et al. [17] have documented by ChIP (chromatin immunoprecipitation) analysis that C/EBPβ binds to the asparagine synthetase AARE in vivo and thus functions as an important component of the AAR pathway.

The present study was designed to survey other C/EBP family members for amino acid responsiveness and to investigate the mechanism by which C/EBPβ expression is induced in response to activation of the AAR pathway. The results documented that amino-acid-deprivation slowed the turnover rate of the C/EBPβ mRNA slightly, but increased transcription from the C/EBPβ gene by about 8-fold. Transient transfection of genomic fragments linked to a luciferase reporter gene demonstrated that the C/EBPβ promoter plays no major regulatory role with regard to the amino acid responsiveness. However, deletion analysis of a genomic region located 3' downstream to the protein coding sequence of the intronless C/EBPβ gene revealed that a 93 bp fragment contained an amino-acid-responsive activity that functioned as an enhancer and that was required for the induction following AAR pathway activation. Mutagenesis of possible AAREs within the 93 bp region implicated a C/EBP-ATF composite site as a primary contributor, but the data suggest that multiple sites are required. Exogenous expression of ATF4 caused increased transcription from a reporter plasmid containing the 93 bp fragment, and ChIP analysis documented in vivo binding of ATF4 to this enhancer region of the C/EBPβ gene, but not to the promoter. Collectively, the data indicate that the increased transcription seen, following amino-acid-limited, from the C/EBPβ gene is the mechanism by which elevated expression of this key regulator of the AAR pathway occurs.

**MATERIALS AND METHODS**

**Chemicals**

ActD (actinomycin D) was obtained from Sigma−Aldrich (St. Louis, MO, U.S.A.) and a stock solution (300 µM) was prepared in 100% (v/v) ethanol. An aliquot of the stock solution (or the solvent for the control) was added directly to the cells at a final ActD concentration of 5 µM.

**Cell culture**

Human hepatoma HepG2 cells were cultured in MEM (minimal essential medium, Mediatech, Herndon, VA, U.S.A.), pH 7.4, supplemented to contain a 1 × non-essential amino acid solution (Mediatech), 4 mM glutamine, 25 mM NaHCO3, 100 µg/ml streptomycin sulphate, 100 units/ml penicillin G, 0.25 µg/ml amphotericin B and 10% (v/v) FBS (fetal bovine serum) (Gibco/Invitrogen, Carlsbad, CA, U.S.A.). Cells were maintained at 37°C in a CO₂/air (1:19) 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 a medium deficient in either one of two essential amino acids: amino-acid-complete MEM versus MEM lacking histidine (Gibco/Invitrogen), or amino-acid-complete DMEM (Dulbecco’s modified Eagle’s medium, Mediatech) versus DMEM lacking methionine (Gibco/Invitrogen). The induction of C/EBPβ expression after limitation for either of these amino acids was qualitatively similar and mechanistically the same. For the period of amino-acid-deprivation, all media were supplemented with 10% (v/v) dialysed FBS (Sigma).

**RNA isolation and Northern blot analysis**

HepG2 cells were cultured to 70–80% confluence in 60-mm-diameter dishes and then incubated for the indicated time in complete MEM or MEM lacking histidine. Total cellular RNA was isolated using an RNAeasy® Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.). 32P-radiolabelled cDNA probe synthesis and Northern analysis was performed as described by Aslanian et al. [27]. The cDNA probe for C/EBPβ was nt + 1425 to + 1632, which corresponds to a segment of the 3' untranslated region, obtained by PCR and confirmed by sequencing. The cDNA probe for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was the entire protein coding sequence, obtained from Dr Anupam Agarwal, Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, U.S.A.

**Subcloning of the human C/EBPβ gene**

A BAC (bacterial artificial chromosome) clone (RP11-112L6) containing sequence from human chromosome 20 was obtained from the Wellcome Trust Sanger Institute, Hinxton, Cambridge, U.K. To obtain a C/EBPβ-containing genomic fragment, the BAC clone was digested with EcoRI and PvuI, the fragments were separated by preparative field inversion gel-electrophoresis, and then they were ligated into the EcoRI site of the pBluescript® II SK vector (Stratagene, La Jolla, CA, U.S.A.). Using the C/EBPβ cDNA probe described above, colony hybridization was used to screen the resulting DH5α colonies and an 11.5 kb C/EBPβ clone was obtained that contained nt - 8451 to + 3074, relative to the transcription start site (+ 1).

**Deletion analysis**

C/EBPβ fragments containing nt - 8451/+ 157 and - 1595/+ 157 were obtained by restriction endonuclease digestion of the - 8451/+ 3074 clone, whereas the C/EBPβ promoter fragment (nt - 325/+ 157) was prepared by PCR and checked by sequencing. The C/EBPβ sequences + 1554/+ 1646, + 1423/+ 2213 and + 1423/+ 3541, which are 3' to the protein coding sequence, were amplified by PCR using either the - 8451/+ 3074 fragment or the original BAC clone as template. The promoter fragments were checked by sequencing and then ligated into the Smal site, upstream of the firefly luciferase protein coding sequence within the pGL3-basic vector (Promega, Madison, WI, U.S.A.). The C/EBPβ gene downstream sequences were tested for amino acid responsiveness by ligation into the BamHI site, located 3' to the luciferase protein coding sequence. To test the C/EBPβ genomic sequences in conjunction with the SV40 (simian virus 40) promoter, oligonucleotides were synthesized with BamHI linkers (Invitrogen) and ligated into the BamHI site of the pGL3-promoter vector (Promega).
Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quik-
Change® Site-Directed Mutagenesis Kit (Stratagene). Block
substitutions were made within the C/EBPβ 3′ genomic sequence from
nt +1423 to +2213, which was cloned into BamHI site down-
stream of the firefly luciferase reporter gene (pGL3-basic vector,
Promega) under the control of the C/EBPβ promoter fragment
nt −1593/+157. The mutations were confirmed by DNA sequen-
cing. A C/EBP-ATF site (underlined), 5′-TGATGCGAATTC-3′
(nt +1567/+1576), was mutated to 5′-CAGGCGGTAT-3′. An
UPRE (unfolded protein response element; underlined) and
its 3′ flanking sequence, 5′-ACTGACGCAACCCACGTG-3′
(nt +1618/+1627), were changed to 5′-ACTGACCTTGGATAT-
TGG-3′. A portion of the NSRE-2 (nutrient sensing response
element-2) (underlined) and its 3′ flanking nucleotides, 5′-TGG-
AACTGTCAG-3′ (nt +1628/+1639), were replaced by 5′-TGG-
GGACTCAGT-3′. The C/EBPβ wild-type or mutated sequences
were transiently transfected into HepG2 cells, as described below,
and the enhancer activity was assayed by measuring the firefly
luciferase activity.

Transient transfection and luciferase reporter assay

HepG2 cells were transfected at ∼50% confluence in 24-
well plates (2 × 104 cells/well) using the SuperFect® transfection
reagent according to the manufacturer’s instructions (Qiagen,
Valencia, CA, U.S.A.). For each transfection, 1 μg of the pGL3
Firefly luciferase reporter construct, driven by the indicated
promoter, was co-transfected along with 0.5 ng of a reference
Renilla (sea pansy) luciferase expression plasmid, phRL-SV40
(Promega). After transfection and a subsequent 18 h recovery in
sea pansy luciferase expression plasmid, phRL-SV40
Renilla promoter, was co-transfected along with 0.5 ng of a reference
Firefly luciferase reporter construct, driven by the indicated
reagent according to the manufacturer’s instructions (Qiagen,
its 3′ UPRE (unfolded protein response element; underlined) and
AAC
well plates (2
samples containing 30
blot analysis. Protein content was quantified by a Lowry assay and
Immunoblot analysis

After incubation in amino-acid-complete or amino-acid-deficient
medium for 0–24 h, total cell extracts were prepared for immuno-
 blot analysis. Protein content was quantified by a Lowry assay and
samples containing 30 μg of protein were separated on a precast
Criterion™ Tris/HC1 polyacrylamide gel (Bio-Rad, Hercules,
CA, U.S.A.) or a standard 20 cm long 10% gel (for C/EBPβ).
After electrophoresis and transfer to a Bio-Rad nitrocellulose membrane,
the membrane was stained with Fast Green to check for equal
loading and then incubated with 10% blocking solution [10%
(w/v) Carnation non-fat dry milk and Tris-buffered saline/Tween
(30 mM Tris base (pH 7.6), 200 mM NaCl and 0.1% Tween-20)]
for 1 h at room temperature with mixing. Immunoblotting was
performed using rabbit polyclonal antibodies against the α, β,
γ, δ, or ε isoforms of C/EBP (Santa Cruz Biotechnology, Santa
Cruz, CA, U.S.A.) at an antibody concentration of 0.2–2 μg/ml
in 10% dry milk blocking solution for 2 h at room temperature
(∼21°C) with mixing. The membrane was washed five times for
5 min in 5% blocking solution on a shaker and then incubated
with peroxidase-conjugated goat anti-rabbit secondary antibody
(Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) in
5% dry-milk blocking solution at a 1:20000 dilution for 1 h at
room temperature with mixing. The membrane was then washed
two times for 5 min in 5% dry milk blocking solution and two
times for 5 min in TBS/T (30 mM Tris base pH 7.6, 200 mM
NaCl and 0.1% Tween-20). The bound secondary antibody was
detected using an Enhanced Chemiluminescence kit (Amersham
Biosciences, Piscataway, NJ, U.S.A.) and exposing the membrane
to Biomax® MR film (Kodak, Rochester, NY, U.S.A.). To provide
a demonstration of equal loading, some membranes were re-
probed with a 1:5000 dilution of an antibody specific for actin
(Sigma).

ChIP analysis

For ChIP analysis, HepG2 cells were seeded at 1.5 × 105/150-
mm-diameter dish with complete MEM and then grown for 24 h.
Cells were transferred to fresh MEM for 12 h before transfer
to either complete MEM or MEM lacking histidine for the time
period indicated in each Figure. Protein and DNA were cross-
linked by adding formaldehyde directly to the culture medium
to a final concentration of 1%, the reaction being stopped 10 min
later by adding 2 M glycine to a final concentration of 0.125 M.
Cross-linked chromatin was solubilized by sonication using a
Sonic Dismembrator (Model 100, Fisher Scientific Co.) for five
bursts of 40 s at power 5 with 2 min cooling on ice between
each burst. An extract from 1 × 105 HepG2 cells was incubated with
2 μg of antibody. The antibody-bound complex was precip-
itated by Protein A–Sepharose beads (Amersham Biosciences).
The DNA fragments in the immunoprecipitated complex were
released by reversing the cross-linking at 65°C for 5 h and puri-
ified using a QIAquick® PCR purification kit (Qiagen Inc.).
 Purified, immunoprecipitated DNA was analysed by qPCR
(quantitative real-time PCR). Primers for the qPCR were de-
signed using Vector NTI® Version 7.1 software (InforMax Inc.,
Frederick, MD, U.S.A.) to amplify the C/EBPβ proximal pro-
moter sequence nt −411 to −481 (forward primer, 5′-GGGAG-
TCGTCACAGGCCGTCAA-3′, and reverse primer, 5′-TCCCG-
TAACTCCCACCTCTC-3′) and the 3′ genomic sequence at
nt +1607 to +1670 (forward primer, 5′-CGGACACGCAG-
GTAACCTGCA-3′, and reverse primer, 5′-CAGGACACAGG-
CCGTAAGAAC-3′). To measure the transcription rate from
the intronless C/EBPβ gene, the method of Sandoval et al.
[28] was used, which relies on ChIP analysis to monitor Pol II
(RNA polymerase II) binding at a region distal to the promoter.
For this purpose primers were chosen within the protein-
coding region of the gene (forward primer, 5′-AGAACGAGC-
GGCTGCAAGAAG-3′, and reverse primer, 5′-CAATTC-
CAGGGCTGCTC-3′). The qPCR analysis was performed using
the DNA Engine Opticon® 2 system (Genetic Technologies,
Miami, Fl, U.S.A.) and the product was detected with SYBR®
Green I. Serial dilutions of input chromatin were used to
generate a standard curve for determining the relative amount of
product. Duplicate for both the standards and the samples
were simultaneously amplified using the same reaction master
mixture. The reactions were incubated at 95°C for 15 min to activate
the polymerase, followed by amplification at 95°C for 15 s and 60°C
for 60 s for 35 cycles. 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 and results are reported as the means ± S.E.M.

RESULTS

Effect of amino-acid-limitation on the protein content for members
of the C/EBP family

Previous work from our laboratory had shown that the abundance
of C/EBPβ was increased in response to amino-acid-deprivation

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Figure 1 Amino-acid-limitation of HepG2 cells does not affect the protein content of C/EBPα, C/EBPγ or C/EBPδ family members

HepG2 cells were maintained in complete MEM to reach 70–80% confluence and then transferred to fresh complete MEM (MEM) or MEM lacking histidine (MEM - AA) for the period of time indicated. Total cell extracts were isolated and subjected to immunoblotting, as described in the Materials and methods section. After probing the blot with an antibody specific for C/EBPα, C/EBPγ or C/EBPδ the membrane was probed with an antibody against actin to ensure equal loading between lanes. A representative blot for each protein is shown, but analysis was performed on multiple cell preparations with qualitatively similar results.

of HepG2 cells [17]. The CHOP gene is also known to be amino-acid-regulated [29]. Other members of the C/EBP family were surveyed for the response to amino-acid-limitation by using a total cell protein extract to immunoblot for each member of the family. Figure 1 shows that C/EBPα, C/EBPγ and C/EBPδ exhibited only minor changes in their abundance following amino-acid-deprivation. C/EBPε protein was not detectable, consistent with its primary role in myeloid cells. The protein content for the LAP (liver-activating protein) isoform of C/EBPβ was induced by methionine limitation, and the protein was detected as a collection of bands migrating from 40–45 kDa (Figure 2). The multiple bands may be the result of post-translational modification, as the C/EBPβ protein is known to be the target of phosphorylation and SUMOylation (SUMO is small ubiquitin-related modifier 1) [18,21,30]. The C/EBPβ mRNA is subject to translational control, which leads to the synthesis of a truncated isoform, termed "liver-inactivating protein" (LIP), from a downstream methionine codon [19,22]. As shown by the results presented in Figure 2, the LIP isoform is also induced following amino-acid-deprivation of cells, although the kinetics appear to be slightly different from those for LAP in that, by 22 h, the LAP content peaked and may actually have declined slightly, whereas the amount of LIP continued to increase to 22 h.

Effect of histidine deprivation on C/EBPβ mRNA content

To investigate the mechanism responsible for the increased C/EBPβ protein expression, the C/EBPβ mRNA content was assayed in amino-acid-deprived HepG2 cells for 0–12 h (Figure 3). After a lag of about 30 min, the C/EBPβ mRNA level rose steadily over the next 8 h, resulting in an increase of about 9-fold, in the cells lacking the amino acid. Although C/EBPα mRNA content was reported to be increased by amino-acid-limitation of rat hepatoma cells [26], when the same blot shown in Figure 3 was stripped and re-probed for C/EBPα mRNA, the data revealed that the amount of C/EBPα mRNA actually declined slowly over the 12 h tested (results not shown).

Figure 2 Amino-acid-deprivation increases HepG2 cell C/EBPβ protein content

HepG2 cells were maintained in complete MEM to reach 70–80% confluence (time = 0) and then transferred to fresh complete DMEM (DMEM) or DMEM lacking methionine (DMEM - AA) for 4, 10 or 22 h. Total cell extracts were isolated and subjected to immunoblotting as described in the Materials and methods section. Prior to probing the blot with an antibody specific for C/EBPβ, the membrane was stained with Fast Green (‘Protein Stain’) to ensure equal loading between lanes. The C/EBPβ antibody used is against the C-terminus and therefore detects both the LAP and LIP isoforms of the protein (see the text for details). A representative blot is shown, but analysis was performed on several independent cell preparations with similar results. The asterisks denote a non-specific band that has been reported by other laboratories for this antibody (Santa Cruz Biotechnology, catalogue number SC-150).

Figure 3 Amino-acid-limitation increases the C/EBPβ mRNA content of HepG2 cells

HepG2 cells were maintained in complete MEM to reach 70–80% confluence and then transferred to fresh complete MEM (MEM) or MEM lacking histidine (MEM - AA). Total RNA was isolated at the time indicated and Northern-blot analysis (15 µg/lane) was performed to measure the mRNA content for C/EBPβ or GAPDH. The GAPDH was used as a loading control and a negative control for amino-acid-dependent activation. The blots were digitized by using a phosphorimager, followed by quantification. The data were plotted as the normalized mRNA content (C/EBPβ/GAPDH) and the value for cells maintained for 8 h in the MEM - AA condition was set to be 100%.
Induction of C/EBPβ mRNA content by the AAR Is primarily due to increased transcription

The mRNA for the cat-1 cationic amino acid transporter [31], the cell cycle regulatory proteins p21 and p27 [32], and ATF3 (Y-X. Pan and M. S. Kilberg, unpublished work) are stabilized by amino-acid-deprivation. To test for mRNA stability as a possible mechanism for the AAR enhancement of C/EBPβ mRNA, HepG2 cells were incubated in histidine-free MEM for 8 h to induce C/EBPβ mRNA content and then transferred to either fresh histidine-free MEM or complete MEM, both containing 5 μM ActD (Figures 4A and 4B). The results showed that the half-life of C/EBPβ mRNA in the cells incubated without histidine was increased slightly (2.2 versus 1.8 h), although it is not likely that this modest amount can account for the 9-fold increase in the steady-state mRNA (Figure 3). To assay the transcription rate from the C/EBPβ gene, cells were subjected to histidine deprivation for 2, 4 or 8 h and then ChIP analysis was performed to monitor Pol II binding to a region distal to the promoter. Sandoval et al. [28] demonstrated that measurement of Pol II binding within the coding region of a gene reflects the transcription rate. For the C/EBPβ gene, this analysis showed that the transcription rate was increased by about 8-fold in amino-acid-deprived cells relative to control cells (Figure 4C) and, therefore, increased transcription appears to account for most of the elevation in C/EBPβ mRNA following amino acid stress.

Figure 4 The elevation in C/EBPβ mRNA content following AAR activation is primarily due to increased transcription

HepG2 cells were maintained in MEM lacking histidine for 8 h to reach maximal induction of C/EBPβ mRNA (time = 0). The cells were washed, then incubated in fresh amino-acid-complete MEM plus 5 μM Act D (MEM) or fresh MEM lacking histidine in the presence of ActD (MEM - AA). Total RNA was isolated at the time indicated and, for (A) Northern-blot analysis (15 μg/lane) was performed to measure the mRNA content for C/EBPβ and GAPDH (as a loading control). (B) Rather than Northern blotting, qPCR was performed to quantify the mRNA turnover and the data were plotted as the logarithm of mRNA content versus time after transfer to the ActD-containing media. (C) The transcription rate for HepG2 cells incubated in either complete MEM or MEM lacking histidine (MEM - AA) was analysed using the ChIP approach described in the text. The data were plotted as the fold increase relative to the MEM control and each point represents the mean ± S.E.M. for three independent assays, each performed in duplicate.

The C/EBPβ promoter region alone is not sufficient to mediate induction via the AAR

Human C/EBPβ is an intronless gene located on chromosome 20 [33], and relative to the transcription start site (designated as +1), the first of three translation start sites is at nt +206 and the universal translation stop codon is at nt +1243 (Figure 5A). A polyadenylation signal (5′-AATAAA-3′) is located approximately 1.8 kbp downstream from the transcription start site. To investigate the potential role of the proximal promoter region in mediating C/EBPβ gene induction in response to amino-acid-limitation, a fragment (nt −325/+157) corresponding to the proximal promoter was tested (Figure 5B). This promoter fragment resulted in basal transcription, but no increase under the histidine-limited condition. To test the possibility that an AARE was located further upstream, longer genomic fragments (nt −1595/+157 and −8451/+157) were examined, but similar results were obtained, in that neither a 1.7 nor a 8.5 kbp fragment mediated a response to amino-acid-limitation (Figure 5B).

C/EBPβ genomic sequence 3′ to the protein coding region is essential for the AAR activation

Given that the C/EBPβ promoter did not support induction by amino-acid-deprivation and that C/EBPβ is an intronless gene, the 3′ genomic region was tested for amino acid responsiveness (Figure 6). Sequentially deleted 3′ genomic fragments were ligated downstream of the firefly luciferase reporter gene driven by a C/EBPβ promoter fragment containing nt −1595 to +157. The C/EBPβ 3′ sequence from nt +1423 to +3541 mediated inducible reporter gene expression when cells were deprived of histidine. When this 2.1 kbp genomic sequence was deleted from its 3′ end to a 790 bp fragment covering nt +1423 to +2213, the induction was maintained (Figure 6). Further deletion of this fragment to a 93 bp sequence covering nt +1554 to +1646 still resulted in activated transcription, even though the basal rate, measured in amino-acid-competent MEM medium, was increased as well. To test the hypothesis that the 93 bp 3′ genomic fragment could confer amino acid responsiveness to an unrelated promoter, the
The C/EBPβ 5′ upstream region is not sufficient to mediate induction following amino-acid-deprivation. (A) illustrates the human C/EBPβ genomic structure. The transcription start site for the C/EBPβ mRNA is indicated as nucleotide +1 and all other features are labelled accordingly. The protein coding sequence of this intronless gene is labelled as ‘CDS’ and the untranslated region is labelled as ‘UTR’. (B) Transcription driven by the three different C/EBPβ 5′ upstream regions was monitored for the response to histidine deprivation after transient transfection of HepG2 cells with C/EBPβ-luciferase reporter constructs. Approx. 18 h after transfection, the cells were incubated for 12 h in either complete MEM or MEM lacking histidine (MEM - AA) and then extracts were assayed for firefly and Renilla luciferase activity. ‘Relative luciferase activity’ represents the firefly activity normalized for transfection efficiency with Renilla luciferase expression driven by the SV40 promoter. The value of the relative luciferase activity of the C/EBPβ promoter fragment nt −325/+157 in the MEM condition was set to 1. All data are expressed as the mean ± S.D. for triplicate determinations.

ATF4 regulates transcription from the C/EBPβ gene downstream enhancer

Increased expression of the bZIP transcription factor ATF4 is an important factor in the transcriptional activation of target genes of the AAR pathway [12,13,17]. To determine if the C/EBPβ gene was regulated by ATF4, HepG2 cells were transiently co-transfected with an ATF4 expression plasmid and a luciferase reporter plasmid containing the C/EBPβ-luciferase reporter construct. Approx. 48 h after transfection, the cells were incubated for 12 h in either amino-acid-complete DMEM or DMEM lacking methionine (Figure 7). The data showed that the C/EBPβ-promoter-only construct was relatively inert to ATF4. However, when either downstream fragment was present, transcription was increased significantly by ATF4 expres-

 Mutagenesis of the 93 bp C/EBPβ downstream region

Computer analysis revealed that within the 93 bp C/EBPβ 3′ genomic region there is a 9 bp sequence, present at nt +1568 to +1576 (5′-TGATGCAAT-3′), that is identical with the AARE within the genes for CHOP (CHOP/GADD153) [34] and the System A transporter SNAT2 [11]. Sequences similar to this one are
regarded as ‘C/EBP-ATF composite sites’ [35,36] and have been shown to mediate the amino-acid-response pathway through binding of ATF4 [13,17]. For the C/EBPβ gene, this C/EBP-ATF sequence is completely conserved across the human, rat and mouse species [23]. However, mutating the core and flanking nucleotides of the C/EBP-ATF sequence had only a partial effect (7.5-fold versus 3.2-fold) on the induced transcription following amino-acid-deprivation (Figure 8) or on the increased transcription after transient expression of exogenous ATF4 (results not shown). A similar result was obtained when the core sequence was completely deleted from the human C/EBPβ 3′ genomic region (results not shown). In fact, this sequence may have a repressive effect, in that mutation resulted in an increase of transcription to a level 2.1-fold over the MEM control value (results not shown).

To assess transcription factor binding in vivo, HepG2 cells were incubated in complete MEM or MEM lacking histidine for 0–8 h and then ChIP analysis was performed on the C/EBPβ gene (Figure 9). A rabbit anti-chicken IgG was used as the negative control to illustrate the background noise of the assay. When Pol II was immunoprecipitated, a significant level of binding to the AAR activity. ChIP analysis of the C/EBPβ proximal promoter and the downstream genomic region containing enhancer activity

To assess transcription factor binding in vivo, HepG2 cells were incubated in complete MEM or MEM lacking histidine for 0–8 h and then ChIP analysis was performed on the C/EBPβ gene (Figure 9). A rabbit anti-chicken IgG was used as the negative control to illustrate the background noise of the assay. When Pol II was immunoprecipitated, a significant level of binding to the C/EBPβ proximal promoter was detected and the level of that binding was increased after amino-acid-deprivation of the cells (Figure 9). In contrast, when binding for ATF4 was tested for the presence of sequence overlapping with the 93 bp regulatory area of the gene, a basal level of ATF4 binding, well above background, was observed in the amino-acid-replete cells and the amount of ATF4 bound increased by about 6-fold between 2–4 h after histidine deprivation (Figure 10).
The bZIP transcription factors ATF3 as well as C/EBPβ itself have been implicated in the regulation of AARE-containing genes (reviewed in [37]). Consequently, binding of these two factors to the C/EBPβ enhancer region was tested and both were shown to be present. At the AARE within the human ASNS, relative to the rapid increase in ATF4 binding within 45 min after amino acid removal, recruitment of ATF3 and C/EBPβ is delayed several hours, and these two factors are thought to hold in check the level of transcriptional induction [17]. For the C/EBPβ 3′ regulatory region, amino-acid-dependent binding of both ATF3 and C/EBPβ was readily detected (Figure 10), and the kinetics were similar to those for ASNS. That is, a high level of constitutive C/EBPβ binding was observed in cells maintained in amino-acid-complete MEM and, after amino acid removal from the medium, the amount associated with the enhancer steadily increased during the 8 h investigated. The binding of ATF3 in the amino-acid-replete cells was low, but was enhanced by amino-acid-deprivation after a lag of about 4 h (Figure 10). This time course for ATF3 recruitment is consistent with the hypothesis that it is functioning as a transcriptional repressor at the AARE [17], because it coincides with a slow decline in ATF4 binding and with a suppression of the transcription rate from the C/EBPβ gene (Figure 4).

DISCUSSION

The experiments described in the present paper have led to these novel observations. (1) Stabilization of the C/EBPβ mRNA cannot account for the magnitude of the increase in steady-state mRNA following amino-acid-limitation; rather, transcriptional control of the gene appears to be the primary regulatory mechanism. (2) The C/EBPβ 5′ upstream genomic region, up to 8.45 kbp, is not sufficient to induce C/EBPβ expression following amino-acid-limitation. (3) The regulatory element(s) necessary for the induction of C/EBPβ gene are located within a 93 bp genomic sequence that is 3′ to the protein coding sequence. (4) The 93 bp fragment has enhancer-like activity in that it conveys amino acid responsiveness to a heterologous promoter, and it is functional regardless of location and orientation. (5) The amino-acid-responsive fragment of the C/EBPβ gene responds to activation by ATF4. (6) Multiple cis-elements contribute to the activation of the gene. (7) Despite the unique location of the amino-acid-responsive activity, ChIP analysis of the C/EBPβ gene documents a temporal relationship between ATF4, Pol II, C/EBPβ and ATF3 binding that has been seen for other amino-acid-responsive genes [17].

In 1994, Marten et al. [26] reported that, in H4-II-E rat hepatoma cells, the mRNA content for both C/EBPβ and C/EBPα was increased in response to depletion of phenylalanine, methionine, leucine or tryptophan from the culture medium. In the present study, it was established that the C/EBPβ response was similar in HepG2 cells, but in these cells C/EBPα mRNA content actually declined in response to amino-acid-limitation. The difference may be due to cell type, species differences or to experimental
conditions, because Marten et al. incubated their cells in serum-free, amino-acid-depleted medium for 24 h, whereas the present experiments were for 12 h duration or less and the medium contained 10% dialysed FBS. Interestingly, Claeyssens et al. [38] have shown that C/EBPα and C/EBPβ bind to a glutamine responsive element within the promoter of the GAPDH gene. Their results illustrate that members of the C/EBP family also contribute to the transcriptional control of genes induced by the presence of amino acids, in their case, glutamine and certain essential amino acids.

The present results demonstrate that the increase in C/EBPβ mRNA expression following AAR pathway activation is primarily due to increased transcription. Interestingly, this transcriptional activation is not mediated through the C/EBPβ promoter, but rather by a genomic sequence 3′ to the translation stop codon. A few examples of previously identified AARE sequences have been located in the proximal promoter regions of CHOP [7] and ASNS [6], in the first exon of the cat-1 transporter gene [8], and in the first intron of the xCT amino acid transporter gene [9], and [6], in the first exon of the cat-1 transporter gene [8], and [6], in the first intron of the SNAT2 transporter gene [11]. Deletion analysis of the C/EBPβ 3′ genomic sequence demonstrated that the nt +1554 to +1646 sequence contains a regulatory element that activates transcription in response to amino-acid-deprivation and to elevated ATF4. This 93 bp sequence confers amino acid responsiveness to an otherwise inert SV40 promoter and can also function in an orientation- and location-independent manner, suggesting that it contains an enhancer-like activity. Mutagenesis within the 93 bp fragment showed the amino-acid-dependent regulation of the C/EBPβ gene may be complicated in that multiple sites appear to contribute. Further analysis will be necessary to definitively characterize the role of each of these elements.

C/EBPβ has been documented to contribute to the regulation of the human ASNS gene following amino-acid-deprivation, but its exact role is not well understood. By EMSA (electrophoretic mobility-shift assay) and ChIP analysis it was shown that C/EBPβ binds to the AARE in the ASNS gene and that the binding activity is increased in amino-acid-deprived HepG2 cells [16,17]. Furthermore, overexpression of the naturally occurring dominant-negative isoform of C/EBPβ, LIP, blocks the increase in transcription from the ASNS promoter [16]. Overexpression of the C/EBPβ LAP isoform is more complex. Overexpression of C/EBPβ LAP by itself causes induction of basal transcription from the ASNS promoter and further induction of the enhanced transcription after amino-acid-deprivation [16]. However, when co-expressed with ATF4 and ATF3, C/EBPβ appears to act in concert with ATF3 to hold in check the induction of the ASNS gene by ATF4 [17]. Accordingly, after amino-acid-deprivation, increased expression of endogenous C/EBPβ and its binding to the ASNS promoter in vivo peaks at a time when the transcription rate from the ASNS gene is beginning to subside [17]. This pattern of C/EBPβ and ATF3 binding was also observed in the present study at the C/EBPβ gene 3′ regulatory region. Thus future studies aimed at understanding the control of expression from the C/EBPβ gene itself and the function of C/EBPβ at AARE sites within a number of genes will be important in advancing our understanding of the AAR pathway.

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