Activation of the *ATF3* gene through a co-ordinated amino acid-sensing response programme that controls transcriptional regulation of responsive genes following amino acid limitation

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Expression of ATF3 (activating transcription factor 3) is induced by a variety of environmental stress conditions, including nutrient limitation. In the present study, we demonstrate that the increase in *ATF3* mRNA content following amino acid limitation of human HepG2 hepatoma cells is dependent on transcriptional activation of the *ATF3* gene, through a highly co-ordinated amino acid-responsive programme of transcription factor synthesis and action. Studies using transient over-expression and knockout fibroblasts showed that several ATF and C/EBP (CCAAT/enhancer-binding protein) family members contribute to *ATF3* regulation. Promoter analysis showed that a C/EBP-ATF composite site at –23 to –15 bp relative to the transcription start site of the *ATF3* gene functions as an AARE (amino acid response element). Chromatin immunoprecipitation demonstrated that amino acid limitation increased ATF4, ATF3, and C/EBP β binding to the *ATF3* promoter, but the kinetics of each was markedly different. Immediately following histidine

removal, there was a rapid increase in histone H3 acetylation prior to an enhancement in ATF4 binding and in histone H4 acetylation. These latter changes closely paralleled the initial increase in RNA pol II (RNA polymerase II) binding to the promoter and in the transcription rate from the *ATF3* gene. The increase in ATF3 and C/EBP β binding was considerably slower and more closely correlated with a decline in transcription rate. A comparison of the recruitment patterns between ATF and C/EBP transcription factors and RNA polymerase II at the AARE of several amino acid-responsive genes revealed that a highly co-ordinated response programme controls the transcriptional activation of these genes following amino acid limitation.

Key words: amino acid response element (AARE), amino acid-sensing, activating transcription factor 3 (ATF3), CCAAT/enhancer-binding protein (C/EBP), histone acetylation, transcription regulation.

INTRODUCTION

Transcriptional regulation in eukaryotes requires the co-ordinated recruitment of RNA Pol II (RNA polymerase II) along with a variety of factors such as activators, repressors, mediators, chromatin-remodelling factors and other chromatin-associated proteins [1]. Nutrient control of gene expression in mammalian cells is an important aspect of regulating cellular responses to changes in the environment. Limitation of amino acids to mammalian cells modulates protein expression at the transcriptional [2], post-transcriptional [3–5], and translational [6,7] levels. The signal transduction pathway that initiates these mechanisms is referred to as the AAR (amino acid response). ATF (activating transcription factor) and C/EBP (CCAAT/enhancer-binding protein) proteins are subfamilies of the larger bZIP (basic region/leucine zipper) transcription factor family, which also includes members of the Jun/Fos family [8]. ATF4 is an ATF family member that is expressed in a variety of tissues and tumour cell lines [8]. Translation from pre-existing *ATF4* mRNA is enhanced in stress conditions that lead to eIF2 α (eukaryotic initiation factor 2 α) phosphorylation, including amino acid deprivation [9], ER (endoplasmic reticulum) stress [10], the presence of long double-stranded RNA [11] and haem deficiency [12]. Both transcription

[13] and translation [7,14] of ATF4 are selectively increased in stress conditions, even when global protein synthesis is repressed, resulting in the induction of ATF4 target genes such as *ASNS* (asparagine synthetase) [13,15], *CHOP* (C/EBP homology protein) [16], and another member of the ATF family, *ATF3* [17–20].

ATF3 is expressed at low levels in normal and quiescent cells, but can be rapidly induced in response to diverse stress signals and is likely to be involved in controlling a wide variety of cellular activities [21]. Pan et al. [19] and Jiang et al. [20] demonstrated that the expression of ATF3 is increased in response to either amino acid deprivation or to ER stress, by mechanisms requiring the eIF2 α kinases GCN2 and PERK respectively [20]. The human *ATF3* gene structure has been studied by Hai et al. [22–24], which revealed a consensus TATA element at around –30 bp of the 5'-flanking sequence relative to the transcription start site, a consensus ATF/CRE (cAMP-response element) site at –93 to –85 bp (5'-TTACGTCAG-3'), and a C/EBP-ATF composite site at –23 to –15 bp (5'-TGATGCAAC-3'). The C/EBP-ATF composite element, so named because it is composed of a half site for each of these two bZIP families [25], has been identified by Hai et al. [24] as the element responsible for the auto-regulation of ATF3. Studies on the induction of ATF3 under stress conditions

Abbreviations used: AAR, amino acid response; AARE, amino acid response element; ASNS, asparagine synthetase; ATF, activating transcription factor; bZIP, basic region/leucine zipper; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homology protein; CRE, cAMP-response element; eIF2 α , eukaryotic initiation factor 2 α ; EMSA, electrophoresis mobility shift analysis; ER, endoplasmic reticulum; FBS, fetal bovine serum; HH3, histone H3; MEF, mouse embryonic fibroblast; MEM, minimal essential medium; qPCR, quantitative PCR; RNA Pol II, RNA polymerase II; RT, reverse transcriptase; SNAT2, sodium-coupled neutral amino acid transporter-2; VEGF, vascular endothelial growth factor.

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have just begun to reveal the full scope of regulatory mechanisms. Ron et al. [15] have shown by microarray analysis that in *atf4* knockout MEF (mouse embryonic fibroblast) cells, tunicamycin-induced *ATF3* mRNA expression was greatly reduced compared with wild-type cells. Wek et al. [20] have shown that mutation of *atf4* and the eIF2 α kinases, *gcn2* or *perk*, greatly impaired induction of ATF3 following amino acid limitation, and the authors concluded that ATF3 is integral to the stress response mediated by these eIF2 α kinases. The sequence of the C/EBP-ATF composite element (5'-TGATGCAAC-3') within the *ATF3* promoter differs by one nucleotide (underlined) from those C/EBP-ATF composite sites that function as AAREs (amino acid response elements) in the *ASNS* (5'-TGATGAAAC-3') and *CHOP* (3'-TGATGCAAT-5') promoters [26,27]. These previous reports support the hypothesis that expression from the *ATF3* gene is regulated by ATF4, but possible transcriptional regulatory element(s) have yet to be identified, and a direct demonstration of ATF4 binding to the *ATF3* promoter following amino acid limitation has not been reported.

The present study aimed to investigate the molecular mechanism for transcriptional activation of the *ATF3* gene following amino acid limitation. Promoter analysis showed that the C/EBP-ATF composite site at -23 to -15 bp of the *ATF3* promoter region is a functional AARE. Transient over-expression showed that several ATF and C/EBP family members contribute to the regulation of *ATF3* transcription, and amino acid limitation in MEF knockout cells confirmed these observations. ChIP (chromatin immunoprecipitation) analysis established that ATF4 directly binds the *ATF3* promoter and activates transcription in a time-dependent manner following histidine limitation. The results reveal that specific temporal interactions of ATF4, ATF3 and C/EBP β with the *ATF3* promoter control the transcription rate of the *ATF3* gene. An increase of HH3 (histone H3) and HH4 acetylation status within the *ATF3* promoter region immediately following amino acid limitation closely correlated with the change in transcription rate. As an extension of the ATF3 data, these relationships were investigated in a number of other genes containing AARE sites and the results demonstrate that following amino acid limitation there is a similar response for the transcriptional activation of many genes. The response is composed of a sequential modification of chromatin, binding of specific bZIP transcription factors and recruitment of RNA Pol II.

MATERIALS AND METHODS

Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.): ATF1, sc-270; ATF2, sc-187; ATF3, sc-188; ATF4, sc-200; C/EBP α , sc-61; C/EBP β , sc-150; RNA Pol II, sc-899. Acetylated HH3 (06-599, specific for acetylated Lys⁹ and Lys¹⁴) and acetylated HH4 (06-866, recognizes acetylated H4 at Lys⁴, Lys⁷, Lys¹¹ and Lys¹⁵) antibodies were purchased from Upstate Biotechnology (Charlottesville, VA, U.S.A.).

Transcription factor expression clones

The expression plasmids for ATF3, ATF4, C/EBP α and C/EBP β were provided generously by Dr T. Hai (Molecular and Cellular Biochemistry, Ohio State University, Ohio, OH, U.S.A.), Dr Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA, U.S.A.), Dr P. Johnson (National Cancer Institute) and Dr H. Nick (University of Florida) respectively.

MEF cell lines

Wild-type, mutated and knockout MEF cells were provided by the following researchers: Dr H. P. Harding and Dr D. Ron (Skirball Institute, New York University School of Medicine, New York, NY, U.S.A.; *gcn2*); Dr D. Scheuner, Dr J. Mitchell and Dr R. Kaufman (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI, U.S.A.; *eif2 α*); Dr T. Hai (*atf3*); Prof. T. Townes (Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL, U.S.A.; *atf4*); Dr G. Darlington (Texas Medical Center Digestive Diseases Center, Baylor College of Medicine, Houston, TX, U.S.A.; *c/ebp α*), Dr P. Johnson (NIH; *c/ebp β*); and Dr Q. Ma and Dr P. LuValle (Department of Anatomy and Cell Biology, University of Florida, Gainesville, FL, U.S.A.; *atf2*). MEF cells were cultured in Dulbecco's modified Eagle's medium with glutamine (Mediatech, Herndon, VA, U.S.A.), supplemented with 1 mM non-essential amino acids, 55 μ M 2-mercaptoethanol, 10% (v/v) FBS (fetal bovine serum), 100 μ g/ml streptomycin sulfate, 100 units/ml penicillin G and 0.25 μ g/ml amphotericin B.

Cell culture

Human HepG2 cells were cultured in MEM (minimal essential medium; pH 7.4; Mediatech), with 1 $\times$ non-essential amino acid solution, 4 mM glutamine, 100 μ g/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 μ g/ml amphotericin B and 10% (v/v) FBS. Cells were maintained at 37°C in a 5% CO₂/95% air incubator, and were replenished with fresh MEM and serum 12 h prior to initiating all treatments. Nutrient deprivation was performed by incubating the cells in complete MEM or MEM lacking histidine, each containing 10% (v/v) dialysed FBS. When inhibitors were used, an aliquot of a stock solution or the solvent for the control was added directly to the incubation medium.

RNA isolation and real-time RT (reverse transcriptase)-PCR

Total cellular RNA was isolated from HepG2 cells using the Qiagen RNeasy Kit (Qiagen, Valencia, CA, U.S.A.). For each sample, 2 μ g of RNA was dried and resuspended in RNase-free H₂O to a final concentration of 200 ng/ μ l. To measure the relative amount of the *ATF3* mRNA, real-time RT-PCR analysis was performed using a DNA Engine Opticon 2 system (MJ Research, Reno, NV, U.S.A.) and detection with SYBR Green I, as described previously [19]. The primers for the amplification were: sense 5'-TGATGCTTCAACACCCAGGCC-3' and anti-sense 5'-AGGGGACGATGGCAGAAGCA-3'. The reactions, containing 200 ng total RNA, were incubated at 50°C for 30 min followed by 95°C for 15 min to activate the Taq polymerase, and then amplified 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°C to 95°C to ensure that a single product was amplified in the reaction. Samples from at least three independent experiments were analysed in duplicate and the results expressed as the means $\pm$ S.E.M.

Transcription rate determination

A ChIP procedure, using the presence of RNA Pol II within the coding region of the target gene as a way to measure transcription initiation and elongation [28], was used to monitor the transcriptional activity from the *ATF3* gene. Primers were used to generate an amplicon from well within the coding region (exon E [23]) of the *ATF3* gene to avoid interference with RNA Pol II paused at the promoter (sense primer, 5'-GCTGTCACCACGTGCAGTATGTCA-3' and anti-sense primer,

5'-CTGTTCTCCTCTTGCTGACAAGC-3'). The reactions were incubated at 50°C for 2 min followed by 95°C for 15 min to activate the Taq polymerase and amplification for 35 cycles at 95°C for 15 s, and 58°C for 60 s. After qPCR (quantitative PCR), melting curves were acquired by stepwise increase of the temperature from 55°C to 95°C to ensure that a single product was amplified in the reaction.

Transfection and reporter assay of *ATF3* genomic constructs

The reporter constructs used to measure *ATF3* promoter activity were made as follows: *ATF3* genomic fragments, containing –107 to +35 bp or –59 to +35 bp relative to the transcription start site, were obtained by PCR from genomic DNA and then cloned into the KpnI site of the promoter-less pGL3 vector containing Firefly luciferase as the reporter gene (Promega, Madison, WI, U.S.A.), and the integrity verified by sequencing. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA, U.S.A.). Block replacement of the C/EBP-ATF site, 5'-TGATGCAAC-3' with 5'-CAGCGTGGT-3', was made within the *ATF3* –59 to +35 bp genomic sequence. For the *ATF3* promoter–luciferase reporter assays, HepG2 cells were seeded at a density of 1.5×10^5 cells/well in 24-well plates in a total volume of 1 ml/well of complete MEM plus 10% (v/v) FBS, and transfected 18 h later using Superfect reagent (Qiagen) at a ratio of 6 μ l Superfect to 1 μ g of DNA. For each transfection, 0.5 μ g of the *ATF3* promoter–luciferase reporter plasmid was used along with 0.1 μ g of the transcription factor expression plasmids. The total amount of transfected DNA was kept constant among experimental groups by the addition of empty pcDNA3.1 plasmid. Cell extracts were collected 36 h later and the luciferase activity assayed using the Luciferase Reporter Assay System (Promega). If the cells were subjected to amino acid limitation, 18 h after transfection they were placed in either complete MEM or histidine-free MEM for an additional 12 h prior to isolating cell extracts.

ChIP analysis

ChIP analysis was performed according to a protocol published previously [29]. A rabbit anti-chicken IgG was used as the non-specific antibody control. Purified, immunoprecipitated DNA was analysed for *ATF3* sequence by regular PCR and visualized by staining after gel electrophoresis (forward primer, nt –59, 5'-GC-CGCCAGCCTGAGGGCTAT-3'; and reverse primer, nt +35, 5'-CGAGAGAAGAGAGCTGTGCA-3'). The results were quantified by qPCR using an MJ Research DNA Engine Opticon 2 system with detection by SYBR green I. Serial dilutions (4-fold) of input chromatin were used to generate a standard curve for determining the relative amount of products. The standards and the samples, in duplicate, were simultaneously amplified using the same reaction master mixture. Primers were used to amplify genomic sequences surrounding the AARE sequences (all positions are relative to the transcription start site of the gene) in: *ASNS* (–87 to –22 bp); *ATF3* (–59 to +35 bp); *C/EBP β* (+1607 to +1671); *CHOP* (–216 to –159); *SNAT2* (sodium-coupled neutral amino acid transporter-2; +672 to +745 bp); or *VEGF* (vascular endothelial growth factor; +1626 to +1705 bp).

RESULTS

Effect of amino acid deprivation on steady-state *ATF3* mRNA content and transcription rate

HepG2 human cells were incubated in histidine-free MEM for 0–8 h and the mRNA level for *ATF3* was measured for three independent experiments by real time RT-PCR (Figure 1A). An

Figure 1 Effect of amino acid limitation on steady-state *ATF3* mRNA content and transcription rate

HepG2 cells were incubated for 0–8 h in complete MEM or in MEM lacking histidine (MEM – His). (A) At the times indicated, RNA was isolated and analysed in duplicate by real time RT-PCR for *ATF3* and the ribosomal protein L7a mRNA content. The data were presented as the ratio of *ATF3* mRNA to L7a control and the graph shows means $\pm$ S.E.M. of three independent experiments. (B) To assay the transcription rate from the *ATF3* gene, binding of RNA Pol II within the coding region was measured, as described in the Materials and methods section. The results are means $\pm$ S.E.M. of three independent experiments measured in duplicate.

initial increase in *ATF3* mRNA content was observed after 2 h of histidine deprivation and reached a value of about 15-times higher than the control after 8 h. To investigate whether transcription contributed to the increase of *ATF3* mRNA, HepG2 cells were incubated in histidine-free MEM for 0–8 h and transcriptional activity was measured by ChIP analysis of RNA Pol II binding within the protein-coding region [28]. This analysis showed that after 2 h the transcription rate was increased approx. 2-fold in amino acid deprived cells compared with the control cells (Figure 1B). Previous analysis of *ATF3* mRNA turnover had revealed that the half-life was increased in the histidine-deprived state [30]. The present results documented that increased transcription also contributes to the elevation in *ATF3* mRNA following amino acid limitation, and the difference in absolute magnitude between the increase in transcription rate and the steady-state mRNA can be explained by the amino acid-dependent stabilization of the *ATF3* mRNA.

Expression level of *ATF3* in MEF cells following amino acid limitation

The activities associated with GCN2, eIF2 α , ATF4, ATF2 and C/EBPs are reported to be components of the AAR pathway (reviewed in [31]). The expression level of *atf3* mRNA was measured in wild-type MEF and MEF cells deficient for these factors, after subjecting them to histidine limitation (Figures 2A–2F). *atf3* mRNA level was enhanced in wild-type MEF cells deprived of histidine, although the degree of the increase varied among the wild-type fibroblasts, each independently isolated by different laboratories. The increase in *atf3* mRNA was diminished to varying degrees in MEF cells devoid of *gcn2*, *atf4*, *atf2*, *c/ebp α* , or those expressing an eIF2 α ^{S51A} mutant (Figures 2A–2E), suggesting that each of these factors is contributing to the activation of the *atf3* gene. In contrast, the increased expression

Figure 2 Expression level of *atf3* mRNA in MEF cells lacking AAR pathway mediators

MEF cells were incubated for 8 h in MEM or in MEM lacking histidine (MEM – His) and then RNA was isolated and analysed in duplicate by real time RT-PCR for *atf3* mRNA content (A–F). Wild-type and cells deficient for *gcn2*, *atf4*, *atf2*, *c/ebpα*, *c/ebpβ* or expressing an *eif2α*^{S51A} mutant, were investigated. The graphs show means ± S.E.M. of three independent experiments.

level of *atf3* mRNA after amino acid deprivation was further enhanced in *c/ebpβ* knockout cells (Figure 2F), indicating that C/EBPβ may actually suppress the expression of *atf3* mRNA in wild-type MEF cells following amino acid limitation. This observation is consistent with the results described below, showing that, when present together, the combination of C/EBPβ and ATF3 antagonizes the ATF4-mediated activation of the *ATF3* promoter (Figure 4B).

The *ATF3* promoter responds to amino acid limitation

To investigate further the transcriptional contribution to the amino acid-dependent increase in *ATF3* mRNA, 107 bp or 59 bp of the 5' upstream genomic region of the human *ATF3* gene was used to drive expression of the Firefly luciferase reporter gene, in an assay for the response to amino acid deprivation (Figure 3A). The constructs contained one or both of two *ATF3* genomic elements, the ATF/CRE site (–93 to –85 bp; 5'-TTACGTCAG-3') and the C/EBP-ATF site (–23 to –15 bp; 5'-TGATGCAAC-3'), both of which can bind specific ATF and C/EBP family members *in vitro* [24]. The –107 to +35 bp and –59 to +35 bp *ATF3* promoter fragments mediated both basal transcription and an induced response to amino acid deprivation, the latter of which was approximately 2- to 3-fold greater than the basal level (Figure 3B), which is consistent with the 2-fold increase in the transcription rate (Figure 1B). The retention of a positive result with the shorter sequence indicates that the ATF/CRE site is not

Figure 3 Transcription driven by the *ATF3* proximal promoter is enhanced by amino acid limitation

HepG2 cells, at approx. 60% confluence, were transfected with plasmid constructs containing the indicated 5'-upstream genomic region of human *ATF3*, linked to the Firefly luciferase reporter gene in pGL3. (A) The –59/+35 fragment was tested as the native sequence or with the C/EBP-ATF composite site (–23 to 15 bp) block mutated (–59/+35 mutant). (B) After transfection the cells were incubated for 18 h and then placed for an additional 12 h in either complete MEM or histidine-free MEM prior to isolation of cell extracts and analysis of the Firefly luciferase activity. For each condition within an experiment, assays were performed in quadruplicate. The results are means ± S.D. from three independent experiments.

required for the amino acid response by the *ATF3* promoter. The –59 to +35 bp *ATF3* genomic fragment contains the C/EBP-ATF site that is analogous to other known sequences that have AARE activity [31]. As further proof of its role, block replacement of the C/EBP-ATF composite site, in the context of the –59 to +35 bp *ATF3* fragment caused the complete loss of amino acid-regulated promoter activity (Figure 3B).

Regulation of *ATF3* promoter activity by specific ATF and C/EBP family members

HepG2 cells were co-transfected with the Firefly reporter gene driven by the *ATF3* genomic fragments –107 to +35 bp, or –59 to +35 bp, along with an expression vector for the regulatory factors ATF3, ATF4, C/EBPα, or C/EBPβ (Figure 4A) identified in the present study or known to mediate the amino acid-dependent regulation of other genes (reviewed in [31]). Transfection of HepG2 cells with *ATF3* resulted in repression of the basal *ATF3* promoter activity from both genomic fragments, whereas expression of exogenous ATF4, C/EBPα or C/EBPβ enhanced activity from both promoter fragments. The action of these transcription factors on the –59 to +35 bp fragment demonstrates that the ATF/CRE site is not required for their action and is

Figure 4 Regulation of *ATF3* promoter activity by specific ATF and C/EBP family members

HepG2 cells were seeded at 1.5×10^5 /well in a 24-well plate and 18 h later co-transfected with 0.5 μ g of *ATF3* (–107/+35 or –59/+35) promoter/Firefly luciferase reporter plasmid and with 0.1 μ g of an expression plasmid containing the cDNA for *ATF3*, *ATF4*, *C/EBP α* or *C/EBP β* , each driven by the cytomegalovirus promoter. (A) shows the effect of individual factors, whereas (B) shows the results of expression in combination. The total amount of transfected DNA was kept constant among experimental groups by the addition of empty pcDNA3.1 plasmid. Cell extracts were assayed for luciferase activity 36 h after transfection. The results are means $\pm$ S.E.M. ($n = 4$). The inset panel in (A) expands the Luciferase values for the control and *ATF3*-expressing cells to show no significant effect of *ATF3* alone. In (A), the asterisks (*) indicate statistical significance ($P \leq 0.05$) for the value for the –59/+35 fragment, relative to the value for the –107/+35 fragment. In (B), the asterisks (*) indicate $P \leq 0.05$ for the value relative to the control.

consistent with the deletion analysis shown in Figure 3, documenting that the C/EBP-ATF composite site acts as an AARE. However, a significant decrease in the degree of activation by *ATF4* and *C/EBP α* did occur when the –107 to +35 bp fragment was shortened to the –59 to +35 bp sequence (Figure 4A), suggesting that the full effect of these two factors may be influenced by the ATF/CRE site or other sequences between the –107 and –59 bp positions. The suppression of transcription by exogenous *ATF3* is consistent with the results of Wolfgang et al. [24] and indicates that this auto-repression is mediated through the C/EBP-ATF composite site. *ATF3* is also known to repress

transcription from the *CHOP* gene [17,25] and the *ASNS* gene [19,29] via their C/EBP-ATF composite sites.

Previous studies have demonstrated that transient expression of *ATF4*, *C/EBP β* and *ATF3* results in co-ordinately regulated transcription from a reporter gene driven by the *ASNS* promoter [32]. Although the MEF knockout cells and the over-expression results for *ATF2* and *C/EBP α* suggested that these factors are required for amino acid dependent regulation of the *ATF3* gene, the ChIP analysis results, shown below, indicated that *ATF2* and *C/EBP α* may constitutively bind to the promoter in both amino acid complete and amino acid deprived conditions. Therefore, control of the *ATF3* gene by combinations of *ATF4*, *ATF3* and *C/EBP β* was investigated further by transiently expressing these factors (Figure 4B). Although expression of *C/EBP β* alone produced a 3-fold increase in *ATF3*-driven transcription, co-expression of *C/EBP β* and *ATF4* together resulted in only a slight enhancement over *ATF4* alone. Conversely, co-transfection of *ATF3* alone or in combination with either *ATF4* or *C/EBP β* resulted in a suppression of transcription. When all three factors were expressed simultaneously, the expression of *ATF3* and *C/EBP β* together resulted in a greater degree of inhibition of the *ATF4*-induced transcription compared with *ATF3* alone (Figure 4B), which indicates that *ATF3* and *C/EBP β* act together to suppress *ATF3* transcription.

Interaction of ATF/CEBP proteins with the *ATF3* promoter *in vivo*

Because transient expression and MEF cells provided evidence for a role of selected ATF and C/EBP proteins in *ATF3* regulation, ChIP assays were performed to confirm whether these factors bind to the human *ATF3* promoter *in vivo*. Chromatin fragments were isolated from HepG2 cells incubated in MEM or histidine-free MEM for 8 h, immunoprecipitated with factor-specific antibodies, and then the immunoprecipitate was tested for *ATF3* promoter sequence (–59 to +35 bp). Gel analysis of the PCR product from of the input DNA produced only a single band with the expected size of 94 bp and immunoprecipitation with a non-specific rabbit anti-chicken IgG antibody, used as negative control in each experiment, did not produce a PCR product (Figure 5A). PCR using immunoprecipitates generated with antibodies specific for *ATF2*, *ATF3*, *ATF4*, *C/EBP α* and *C/EBP β* also amplified the expected *ATF3*-promoter fragment, demonstrating *in vivo* binding of these transcription factors. In contrast, *ATF1* binding was not statistically different from the non-specific IgG control (Figure 5). The initial ChIP experiments, shown as gel bands in Figure 5(A), were repeated and the immunoprecipitates analysed by qPCR (Figure 5B). When immunoprecipitates from cells incubated for 8 h in the absence of histidine were analysed, the absolute amount of *ATF3*, *ATF4* and *C/EBP β* binding was greater than in control (MEM) cells. In contrast, although *ATF2* and *C/EBP α* binding was clearly well above the background, it was unchanged by amino acid limitation. Binding of RNA Pol II increased 2-fold following histidine deprivation (Figure 5B), which is a value that corresponds well to the increase in promoter activity observed in the functional assays (Figure 3B).

Acetylation of HH3 and HH4, and *ATF4* binding, occurs rapidly following activation of the *ATF3* gene

Histone acetylation is associated with increased gene transcription, and hence with transcriptionally active chromatin domains, presumably by conferring accessibility of the DNA template to the transcriptional machinery for gene expression [33,34]. To test for the effect of amino acid limitation on chromatin remodelling at the *ATF3* promoter, ChIP assays were performed over a time course of 0–120 min with antibodies specific for histone

Figure 5 *In vivo* binding of specific transcription factors to the promoter region of the *ATF3* gene

(A) HepG2 cells were incubated with either complete MEM (M) or MEM lacking histidine (H or MEM-His) for 8 h. ChIP assays were performed using antibodies specific for the indicated proteins. A rabbit anti-chicken IgG was used as the negative control (n/s IgG). Using the immunoprecipitated DNA as template, PCR products amplifying -59 to +35 bp of the *ATF3* gene were analysed by agarose-gel electrophoresis (A), or analysed by qPCR (B). The quantified results were plotted as the ratio to a 1:25 dilution of input DNA and presented as means $\pm$ S.E.M. of three independent experiments. * Statistical significance ($P < 0.05$) of the histidine-deprived values compared with the values in the MEM-incubated cells.

modifications associated with enhanced gene transcription: HH3 is acetylated at Lys⁹ and Lys¹⁴, and HH4 is acetylated at Lys⁴, Lys⁷, Lys¹¹ and Lys¹⁵. Figure 6 shows that amino acid limitation caused a time-dependent increase in the acetylation of HH3 at the *ATF3* promoter region, which exhibited a significantly elevated level within 30 min following amino acid limitation, peaked at 45 min and then declined slightly, but was maintained at a higher level than the MEM control over the entire 120 min (Figure 6A). Furthermore, it appeared that these events occurred slightly prior to the acetylation of HH4 and ATF4 binding to the promoter, which paralleled each other, peaking at 60 min following amino acid limitation (Figure 6A). Recruitment of RNA

Figure 6 Acetylation of HH3 and HH4, and concomitant ATF4 and RNA Pol II binding, following activation of the *ATF3* gene

HepG2 cells were incubated in complete MEM (○) or MEM lacking histidine (●) for 0–120 min. ChIP analysis was performed using antibodies specific for ATF4, RNA Pol II or acetylated HH3 or HH4. (A) Results were plotted as the ratio to the value obtained with a 1:25 dilution of input DNA. Results are means $\pm$ S.E.M. of three independent experiments, measured in duplicate. (B) The abundance of ATF4 protein content in nuclear extracts following amino acid deprivation was determined over the 0–120 min incubation in MEM lacking histidine (MEM-His) and a representative autoradiogram is shown.

Pol II also increased between 45 and 60 min. These observations are further supported by immunoblotting showing a rapid nuclear accumulation of newly synthesized ATF4 protein detectable at 30 min following histidine removal (Figure 6B), which correlated well with the ChIP analysis of increased ATF4 binding.

A highly co-ordinated amino acid-sensing response programme

Given the high degree of sequence similarity between the C/EBP-ATF composite site on the *ATF3* promoter and the AARE for other amino acid responsive genes, it is possible that activation of these genes by ATF4 and subsequent suppression by ATF3 and CEBP β may be a general control mechanism. To test this hypothesis, the kinetics for interaction of ATF4, ATF3, CEBP β and RNA Pol II with the AARE regions of known AAR genes, *ASNS* [27,29,35], *C/EBP β* [33], *CHOP* [26], *SNAT2* [36] and *VEGF* [37], were examined by ChIP analysis and qPCR (Figure 7). Some variation in absolute magnitude and the kinetics, especially within the first 4 h, occurred between genes, but in a broader view the association

Figure 7 A highly co-ordinated amino acid-sensing response programme

HepG2 cells were incubated in MEM lacking histidine for 0–8 h and ChIP analysis was performed at 0, 2, 4 and 8 h using the antibodies against ATF4, ATF3, C/EBP β and RNA Pol II. Primers were used to amplify genomic sequences surrounding the AARE sequences in *ASNS* (–87–22), *ATF3* (–59+35), *C/EBP β* (+1607+1671), *CHOP* (–216–159), *SNAT2* (+672+745) or *VEGF* (+1626+1705). Results were plotted as a ratio with the value obtained with a 1:25 dilution of input DNA and normalized against the time = 0 value. Each point represents the mean value of at least two independent experiments, and the variation between ChIP experiments was typically less than 20%.

of ATF4, ATF3, C/EBP β , and RNA Pol II with these genes also revealed a number of similarities in pattern. By 4 h following histidine removal, there was a 3- to 12-fold increase in ATF4 binding that generally paralleled the increased binding of RNA Pol II. The binding of ATF3 was quite variable during the initial 2–4 h, but beyond 4 h ATF3 binding rose relatively sharply for all genes. For C/EBP β , the increased binding began slowly during the 2–4 h period, and then increased steadily between 4 and 8 h (Figure 7). For all of the genes, the times at which ATF3 and C/EBP β binding were the greatest correlated with the plateau in binding of RNA Pol II between 4 and 8 h. Collectively, these data support the hypothesis that a common amino acid-sensing response programme controls the transcriptional regulation of these genes. A similar programme may also be initiated by other stresses that involve phosphorylation of eIF2 α and subsequent ATF4 synthesis, as suggested by the results of Fawcett et al. [25] for arsenite treatment of cells.

DISCUSSION

The present study describes a mechanistic analysis of *ATF3* gene activation following amino acid limitation and also illustrates that a co-ordinated amino acid-sensing response programme controls the transcriptional activation of AARE-containing genes following amino acid limitation. The experiments included in this report have led to the following novel observations. (1) A C/EBP-ATF composite site between –23 and –15 bp relative to the transcription start site of the *ATF3* promoter functions as an AARE to enhance transcription of the gene following amino acid limitation. (2) Over-expression of individual transcription factors documented the ability of ATF3, ATF4, C/EBP α and C/EBP β to regulate *ATF3* transcription. (3) ChIP analysis validated that histidine limitation caused increased *ATF3* promoter binding *in vivo* for ATF3, ATF4, C/EBP β and RNA Pol II, and demonstrated a direct action of these proteins on the *ATF3* gene. (4) Following amino acid limitation, the physical association of ATF4, ATF3, C/EBP β and RNA Pol II with the *ATF3* promoter occurred in a time-dependent manner that was consistent with the observed changes in transcription rate. (5) ChIP analysis also demonstrated that following histidine removal the temporal changes of H3 acetylation at the *ATF3* promoter occurred prior to ATF4 binding.

The 5'-upstream region of the *ATF3* gene contains a previously identified C/EBP-ATF composite site (5'-TGATGCAAC-3'), which is responsible for auto-regulation of the *ATF3* gene [24]. This C/EBP-ATF site differs from the NSRE-1 (nutrient-sensitive response element) core sequence (5'-TGATGAAAC-3') of the *ASNS* gene or the AARE (3'-TGATGCAAT-5') of the *CHOP* gene by only one nucleotide, but both of those changes still permit retention of function by the AARE of *CHOP* [26]. However, whether the presence of this C/EBP-ATF element contributed to the increase in steady-state *ATF3* mRNA following histidine deprivation, and which transcription factors are directly involved in the amino acid-dependent regulation of the *ATF3* gene, were largely unknown prior to this study. Using EMSA (electrophoresis mobility shift analysis), Fawcett et al. [25] demonstrated that ATF3 binds to the C/EBP-ATF site of the *CHOP* gene *in vitro*, and that over-expression of ATF3 repressed both the basal and ATF4-induced transcription from a *CHOP* promoter construct containing this element. Wolfgang et al. [24], also using EMSA, demonstrated that the binding affinity of ATF3 for the C/EBP-ATF sequence was greater than that of ATF1. In the present study, promoter analysis of the *ATF3* gene demonstrated that the C/EBP-ATF composite site functions as an AARE. The transient expression studies involving ATF4, ATF3 and C/EBP β indicate that ATF4 functions as an activator of *ATF3* promoter activity and that subsequently ATF3 has a repressive effect on ATF4 function. Despite the observation that when over-expressed alone C/EBP β causes increased transcription from a reporter plasmid, the data from the *c/ebp β* ^{–/–} MEF cells and co-transfection with ATF3 and ATF4 suggested that C/EBP β can serve as a repressor in concert with ATF3. These results are consistent with our earlier observations [29] showing the ability of ATF3 alone, or C/EBP β in combination with ATF3, to counterbalance ATF4 activation of the *ASNS* gene and, thus, provide transcriptional restraint. In the present study, ChIP analysis verified that histidine limitation caused increased binding of ATF3, ATF4, C/EBP β and RNA Pol II to the *ATF3* promoter region containing the C/EBP-ATF composite site, indicating a direct action of these proteins on the *ATF3* gene. Collectively, the results demonstrate that the C/EBP-ATF composite site functions as an AARE within the *ATF3* promoter and consequently is responsible for the amino acid-dependent regulation of *ATF3* mRNA expression.

Histone acetylation is an important epigenetic modification and evaluation of the *ATF3* promoter in response to amino acid limitation showed that acetylation on HH3 and HH4 is increased in a time-dependent manner. Similar changes also occurred at the *ASNS* promoter [29], but there are distinct differences in acetylation kinetics between the two promoters. The kinetics of HH3 and HH4 acetylation at the *ASNS* promoter were nearly identical to those for ATF4 and RNA Pol II association during amino acid deprivation. The results supported a hypothesis that ATF4 acts as the recruiting factor for an unknown histone acetyltransferase that may make the *ASNS* promoter more accessible to RNA Pol II and the general transcription machinery. However, compared with the *ASNS* promoter, HH3 associated with the *ATF3* promoter appeared to be acetylated earlier, exhibiting a significantly elevated level within 30 min following amino acid limitation and preceding the increased binding of ATF4. The results raise an interesting question with regard to the initial events that make the *ATF3* promoter more accessible to ATF4, RNA Pol II and the general transcription machinery. These results suggest that, although all amino acid responsive genes may have AARE sites that have similar sequences, differential gene expression could be modulated by the level of histone acetylation for each gene. Such differences would permit flexibility between genes with regard to the length and magnitude of transcriptional activation despite the same initial signal. How the degree of histone acetylation is controlled for a given gene is not known.

Chen et al. [29] presented a working model for activation of the human *ASNS* gene in response to amino acid limitation by proposing that ATF4 plays a key role in activating the *ASNS* gene. The model also proposed that there is a self-limiting programme for transcription from *ASNS* that results from a delayed association of transcription factors such as ATF3 and C/EBP β , which appears to suppress the ATF4-induced activation of the *ASNS* gene and make it transitory. The present study extends further the model of Chen et al. [29] by showing that, in general, this sequence of events can be observed for many amino acid responsive genes regulated by amino acid limitation, including *ATF3*, *C/EBP β* , *CHOP*, *SNAT2* and *VEGF*. The results support the hypothesis that following amino acid limitation there is a highly co-ordinated, time-dependent programme of interaction between members of the ATF and C/EBP subfamilies of bZIP transcription factors. While there may well be variations in absolute magnitude and perhaps some differences in the kinetics between individual genes, we propose that this series of events will be the general rule for transcriptional activation of genes by amino acid control and, perhaps, for transcriptional control by another ATF4-mediated stress responses [25].

This research was supported by grants to M. S. K. from the Institute of Diabetes, Digestive and Kidney Diseases, the National Institutes of Health (DK52064, DK70647), and to H. C. from the Leukemia Research Foundation. We thank other members of the laboratory for technical advice, reagents and helpful discussion.

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Received 17 August 2006/14 September 2006; accepted 21 September 2006

Published as BJ Immediate Publication 21 September 2006, doi:10.1042/BJ20061261