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Dynamic changes in genomic histone association and modification during activation of the *ASNS* and *ATF3* genes by amino acid limitation

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Abstract

Amino acid deprivation of mammalian cells triggers several signalling pathways, the AAR (amino acid response), that results in transcriptional activation. For the *ASNS* (asparagine synthetase) and *ATF3* (activating transcription factor 3) genes, increased transcription occurs in conjunction with recruitment of ATF4 to the gene. In HepG2 cells, analysis of the *ASNS* and *ATF3* genes during AAR activation revealed increases in histone H3K4me3 (histone 3 trimethylated Lys⁴) and H4Ac (acetylated histone 4) levels, marks associated with active transcription, but a concurrent loss of total H3 protein near the promoter. The dynamic nature of AAR-regulated transcription was illustrated by a decline in *ASNS* transcription activity within minutes after removal of the AAR stress and a return to basal levels by 2 h. Reversal of *ASNS* transcription occurred in parallel with decreased promoter-associated H4Ac and ATF4 binding. However, the reduction in histone H3 and increase in H3K4me3 were not reversed. In yeast, persistence of H3K4me3 has been proposed to be a ‘memory’ mark of gene activity that alters the responsiveness of the gene, but the time course and magnitude of *ASNS* induction was unaffected when cells were challenged with a second round of AAR activation. The results of the present study document changes in gene-associated nucleosome abundance and histone modifications in response to amino-acid-dependent transcription.

Keywords

activating transcription factor 4 (ATF4); histone acetylation; histone code; histone methylation; nutrient sensing

INTRODUCTION

Imbalances in dietary macronutrients, especially during early developmental stages, are recognized as principle causes for a variety of metabolic disorders that collectively have a significant negative impact on human health (reviewed in [1]). Eukaryotic organisms detect and respond to dietary inadequacy of protein or amino acids via a collection of signalling cascades jointly referred to as the AAR (amino acid response) (reviewed in [2]). The best-characterized of these pathways is initiated by the uncharged tRNA-sensing GCN (general

AUTHOR CONTRIBUTION

Mukundh Balasubramanian helped design the experiments and collected most of the experimental data, and contributed to writing of the paper. Jixiu Shan contributed to the design of the experiments, collected experimental data and helped with editing of the paper. Michael Kilberg helped design the experiments, analysed the data and co-wrote the paper.

control non-derepressible)-2 kinase for which the primary substrate is the α -subunit of the eIF2 (eukaryotic translation initiation factor 2). p-eIF2 α (phospho-eIF2 α) causes suppression of general translation, but paradoxically increases the translation of selected mRNA species, including that for ATF (activating transcription factor) 4 [3]. Microarray analysis has revealed that amino acid deprivation and ATF4 activate hundreds of genes that are involved in functionally diverse physiological processes [4–8]. In response to activation by four different eIF2 α kinases [9], ATF4 binds to target genes via a composite genomic sequence comprising a half-site for C/EBP (CCAAT/enhancer-binding protein) family members and a half-site for ATF members. Wolfgang et al. [10] first annotated a ‘C/EBP–ATF composite site’ in the *CHOP* (C/EBP homology protein) promoter as an ATF3-responsive genomic element [10] and, subsequently, Fawcett et al. [11] further established that, in response to arsenite, there is binding of ATF4 to the site which is subsequently replaced by ATF3 [11]. During the last decade it has been established that there are a number of C/EBP–ATF sequences that lead to a consensus of 5′-TGATGXAAX-3′ and that ATF4–C/EBP β heterodimers activate transcription by binding to these sites in response to a wide variety of stresses, including the unfolded protein response, arsenic, hypoxia and amino acid deprivation (reviewed in [2]). To retain the original nomenclature of ‘C/EBP–ATF’ and to reflect the broad functions of this collection of sequences, we will refer to them collectively as CAREs (C/EBP–ATF response elements). Thus, when the activating stress is amino acid deprivation, these CARE sequences function as AAREs (AAR elements). The *ASNS* (asparagine synthetase) and *ATF3* genes are well-characterized amino-acid-responsive ATF4 targets and within 45 min following amino acid limitation of HepG2 hepatoma cells, ATF4 binds to the CARE sites located in the *ASNS* and *ATF3* proximal promoters [12,13]. ATF4 binding continuously increases for several hours in parallel with the transcription activity, which coincides with increased recruitment of the general transcription machinery, including the TAFs (TATA-binding protein-associated factors) and Pol II (RNA polymerase II). This mechanistic sequence of events for ATF4-dependent transcriptional activation has been documented for many CARE-containing genes [12–14] and indicates that these amino-acid-responsive genes require inducible recruitment of the general transcription machinery triggered by ATF4 binding. The parallel changes that occur in chromatin structure in response to amino acid deprivation are less well understood. Beginning to determine those changes is the purpose of the research on histone modifications described in the present paper.

A number of post-translational covalent modifications of histones have been characterized [15,16]. In particular, acetylation of histone H3 at Lys⁹ and Lys¹⁴ (H3Ac), histone H4 at Lys⁵, Lys⁸, Lys¹² and Lys¹⁶ (H4Ac) [15] and trimethylation of Lys⁴ of histone H3 (H3K4me3) [17] near the transcription start site of genes have correlated with active transcription. Genome-wide studies of nucleosome occupancy have also revealed a depletion of histone H3–H4 tetramers at active regulatory elements of highly transcribed genes, indicative of nucleosome remodelling [18]. In contrast, dimethylation of Lys⁹ of histone H3 (H3K9me2) and trimethylation of Lys²⁷ of histone H3 (H3K27me3) often correlate with the silencing of gene expression [19]. Although the concept that diet influences the mammalian epigenome has been documented for many instances, the exact molecular changes that link specific dietary fluctuations to phenotypic effects is a topic of ongoing research [20]. With regard to dietary protein or amino-acid-induced epigenetic changes, the number of published reports is limited. Lillycrop et al. [21] observed that exposure to a protein-restricted diet in pregnant rats induces fetal DNA hypomethylation of the *PPARA* (peroxisome proliferator-activated receptor α) and *GR* (glucocorticoid receptor) promoters, which leads to an increased hepatic expression in the offspring [21]. Zheng et al. [22] observed that in response to maternal protein restriction during gestation, female offspring exhibited an increase in expression of C/EBP β in skeletal muscle that was associated with an increase in histone H3 and H4 acetylation at the *CEBPB* promoter [22]. Those authors also discovered

that increased histone acetylation as well as methylation of H3K4 at the *GLUT4* (glucose transporter 4) promoter was associated with increased expression in the female offspring [23]. In both cases, neither the histone modifications nor the enhanced transcription was detected in the male offspring. A common aspect of these studies is that epigenetic changes resulting from an imbalanced maternal protein diet leads to altered expression of key transcription factors in the tissue of the offspring, which ultimately causes significant changes in metabolism and other cellular functions.

In addition to these stable chromatin modifications detected *in vivo*, acute studies to monitor epigenetic changes resulting from amino acid deprivation in cell culture have also been reported. Following activation of the AAR, increased acetylation of both histone H3 and H4, in parallel with a rapid increase in ATF4 binding, is observed at the *ASNS* and *ATF3* proximal promoters [13]. In contrast with these two genes for which the CARE element is located just upstream of the transcription start site, the *SNAT2* (sodium-dependent neutral amino acid transporter 2) gene has an intronic CARE enhancer [24]. For the *SNAT2* gene, amino acid deprivation induces H3 acetylation at both the promoter and the downstream CARE, but there is no change in H4 acetylation at either location [25]. Conversely, Bruhat et al. [26] observed an AAR-induced increase in H4 and H2B acetylation at the *CHOP* promoter, but no change in H3 acetylation [26]. Additionally, those authors showed that ATF2, known to encode HAT (histone acetyltransferase) activity [27], mediates the AAR-induced histone acetylation at the *CHOP* and *ATF3* promoters, but not at the *ASNS* promoter. Carraro et al. [28] observed that AAR induction led to an increase in H2B, H3 and H4 acetylation levels at the *TRB3* (tribbles homologue 3) CARE [28]. Collectively, these results document that there are significant differences in the pattern of histone modification at different CARE-containing amino-acid-regulated genes. These differences are likely to have functional consequences with regard to the cellular response to nutrient stress.

The present study was undertaken to address several questions. (i) Are there histone modifications other than acetylation associated with AAR-induced transcription? (ii) What is the status of the histone modifications that were generated in response to amino acid deprivation when the nutrient stress is relieved, i.e. after 'refeeding' of cells? (iii) Do ATF4-induced genes exhibit a period of enhanced sensitivity or refractoriness after a cycle of deprivation and then refeeding? To investigate these gaps in our knowledge, an analysis of the changes in histone modifications within the *ASNS* and *ATF3* genes during the induction and subsequent reversal of the AAR was undertaken. Activation of the stress response caused an increase in H3K4me3 and H4Ac levels concurrent with a loss of H3 protein abundance near the promoters of both genes. The dynamic nature of AAR-inducible transcription was illustrated by the fact that *ASNS* transcription activity was significantly reversed within minutes after removal of the AAR stress and completely reversed to basal levels by 2 h. This decline in *ASNS* transcription occurred in parallel with a decrease in ATF4 binding and in promoter-associated H4Ac. In contrast, both the loss of H3 protein association and the increased H3K4me3 persisted after the removal of the AAR stress. However, the retention of these latter two histone-related changes did not affect the time course or magnitude of *ASNS* transcriptional induction when cells were subjected to a second round of AAR activation.

EXPERIMENTAL

Cell culture

HepG2 cells were cultured in DMEM (Dulbecco's modified Eagle's medium, pH 7.4; Mediatech), supplemented with 1× non-essential amino acids, 2 mM glutamine, 100 µg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 µg/ml amphotericin B and 10 % (v/v) FBS (fetal bovine serum). Cells were maintained at 37 °C in an atmosphere of 5 % CO₂ and

95 % air and maintained in growth phase at 60–70 % confluence. Approximately 12 h before treatments, cells were replenished with fresh DMEM and serum to ensure complete nutrition when experiments were initiated. For AAR induction, cells were incubated in DMEM containing 2 or 5 mM HisOH (histidinol), an amino alcohol that mimics amino acid deprivation. HisOH causes an increase in the amount of uncharged tRNA by competitively inhibiting the charging of histidine on to its corresponding tRNA and thereby inducing the AAR, as described previously [29].

AAR reversal by HisOH washout

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for up to 4 h to activate transcription from the *ASNS* and *ATF3* genes. After this 4 h incubation, cells were incubated with fresh DMEM for 10 min at 37 °C to 'washout' the HisOH and thereby initiate a reversal of the AAR stress. Following this 10 min incubation, cells were then transferred to either fresh DMEM or DMEM containing 2 mM HisOH.

RNA isolation and real-time qPCR (quantitative PCR)

Total RNA was isolated with the TRIzol[®] reagent (Invitrogen) following the manufacturer's recommendations. Steady-state *ASNS* or *ATF3* mRNA levels were assayed by real-time qPCR, as described previously [12]. The amount of short-lived *ASNS* or *ATF3* hnRNA (heterogeneous nuclear RNA), assayed by amplifying across an intron–exon boundary, was used as a measure of transcription activity [30]. The intron–exon primers are given in Supplementary Table S1 at <http://www.BiochemJ.org/bj/449/bj4490219add.htm>. For the HisOH-washout experiments, a 2 μ g aliquot of total RNA was used to synthesize first-strand cDNA with the SuperScript III First-Strand System kit (Invitrogen). qPCR was performed using 2–4 μ l of diluted cDNA mixed with 10 μ l of SYBR Green Master Mix (Applied Biosystems) in a total volume of 20 μ l using forward and reverse primers at a final concentration of 250 nM. The mixture was subjected to 40 cycles of amplification at 95 °C for 15 s, 60 °C for 60 s in a DNA Engine Opticon 3 machine (Bio-Rad Laboratories). The primers used are listed in Supplementary Table S1. After qPCR, melting curves were acquired by stepwise increase from 55 °C to 90 °C to ensure that only a single product was amplified in the reaction. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control and all calculations were based on the difference of C_T (threshold cycle number) of the gene analysed relative to the *GAPDH* mRNA content in the same sample.

Immunoblotting

Whole-cell protein was extracted with 1 $\times$ Laemmli Sample Buffer (Bio-Rad Laboratories) containing 1:20 2-mercaptoethanol (Sigma–Aldrich) and 1 $\times$ Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). A 40 μ g lysate aliquot was separated on a 10.5–14 % Tris/HCl polyacrylamide gel (Bio-Rad Laboratories) and then electrotransferred on to a 0.2 μ m nitrocellulose membrane (Bio-Rad Laboratories). The membrane was stained with Fast Green to check for equal loading and then incubated with blocking solution containing 5 % or 10 % (w/v) Carnation non-fat dried milk in TBST [Tris-buffered saline (30 mM Tris base, pH 7.5, and 200 mM NaCl) and 0.1 % Tween 20] for 1 h at room temperature (23 °C) with rotation. The membrane was then incubated with one of the following antibodies: rabbit anti-ATF4 polyclonal antibody described previously [31]; rabbit anti-p-eIF2 α polyclonal antibody (catalogue number 9721) and rabbit anti-total-eIF2 α polyclonal antibody (catalogue number 9722) from Cell Signaling Technology; or rabbit anti- β -actin polyclonal antibody (catalogue number A2066) from Sigma–Aldrich. The primary antibodies and blots were incubated in blocking solution overnight at 4 °C with rotation. The blots were washed three times for 5 min each in TBST and then incubated with the appropriate peroxidase-conjugated secondary antibody (Kirkegard & Perry Laboratories) for 1 h at room

temperature and then washed five times for 5 min each in TBST. The bound secondary antibody was detected using an ECL (enhanced chemiluminescence) kit (Thermo Scientific) and then exposing the blot to a Classic Blue Autoradiography Film BX (MIDSCI).

ChIP (chromatin immunoprecipitation)

ChIP analysis was performed according to our previously published protocol [12]. HepG2 cells were seeded at a density of 1.5×10^7 per 150 mm dish with complete DMEM and cultured for approximately 36 h, which includes a transfer to fresh DMEM during the final 12 h prior to induction of AAR. Commercial antibodies used were a rabbit anti-Pol II polyclonal antibody (catalogue number sc-899) and, as a non-specific negative control, a normal rabbit IgG (catalogue number sc-2027) purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against total histone H3 (catalogue number ab1791), H3K36me3 (catalogue number ab9050) and H4K8Ac (catalogue number ab15823) were purchased from Abcam. Rabbit anti-histone H3K4me3 monoclonal antibody (catalogue number 04-745), rabbit anti-histone H3K27me3 polyclonal antibody (catalogue number 07-449) and rabbit anti-H4K5,8,12,16 acetylation polyclonal antibody (catalogue number 06-866) were purchased from EMD Millipore. Immunoprecipitated DNA was analysed with qPCR as described above, using primers listed in Supplementary Table S1. All experiments were performed in triplicate to detect experimental variation and were repeated to ensure reproducibility between experiments. The ChIP results are presented as the ratio to input DNA and, where indicated, signals obtained with antibodies against histone modification marks were normalized to the signal obtained with the antibody against total histone H3.

RESULTS

Identification of histone modifications associated with the *ASNS* gene during the AAR

To determine the changes in histone modifications at the *ASNS* gene following amino acid limitation, ChIP experiments were performed to analyse selected histone modification marks that correlate with either permissive or repressive chromatin. Figure 1(A) shows a schematic diagram of the *ASNS* gene, highlighting the ATF4-binding CARE enhancer that mediates the AAR. Also shown are the four locations of the specific regions amplified by PCR for the ChIP experiments. Following AAR induction by a 4 h HisOH treatment, AAR-induced ATF4 recruitment and increased levels of H4Ac were observed at the *ASNS* proximal promoter (Figure 1B). As a negative control, neither ATF4 binding nor the associated H4Ac within the body of the gene was above the non-specific IgG 'background' value (Figure 1B, exon 7). H3K36me3 within the gene body is often indicative of active transcriptional elongation [15]. When H3K36me3 levels were examined at both the *ASNS* promoter and exon 7 following HisOH treatment, only at exon 7 were they significantly above the background and AAR-induced levels (Figure 1B). In contrast, the level of H3K27me3, often enriched at transcriptionally repressed genes [32], was close to the background non-specific IgG antibody levels at the *ASNS* promoter (Figure 1C). As a positive control, the H3K27me3 levels within the promoter region of the *SFRP1* (secreted frizzled-related protein 1) gene, which is epigenetically silenced in HepG2 cells [33], were significantly greater than background (Figure 1C).

The *ASNS* gene was used as a model to investigate H3K4me3, which is often enriched at transcriptionally active genes [32]. Genome-wide analysis of highly transcribed genes has revealed a trend of elevated H3K4me3 levels at approximately 300 bp either side of the TSS (transcription start site) [19]. Transcriptional activity and H3K4me3 levels at the promoter and at + 1 kb of the *ASNS* gene were examined during a 24 h time course after AAR induction (Figure 2). As expected, following AAR induction there was a significant increase in *ASNS* transcriptional activity and steady-state mRNA levels (Figure 2A). Consistent with

the genome-wide data [19], the amount of H3K4me3 did not change at the *ASNS* promoter, but the H3K4me3 levels downstream (+ 1 kb) were significantly increased at 4 and 8 h, after which there was a gradual decline toward basal H3K4me3 levels at 16 and 24 h (Figure 2B). Additional ChIP analysis was performed following a 4 h AAR induction to monitor total histone H3, H3K4me3 and H4Ac levels using the primers denoted in Figure 1(A) to scan the *ASNS* gene at four distinct locations: 1 kb upstream of the TSS (labelled -1 kb), the proximal promoter, 1 kb downstream of the TSS (labelled + 1 kb) and in the transcribed body of the gene (exon 7). Consistent with the results in Figure 2(B), there was a significant increase in H3K4me3 levels at the *ASNS* + 1 kb region following AAR induction and no change in the proximal promoter region (Figure 3A). In addition, there was little or no H3K4me3 signal obtained within the -1 kb or exon 7 regions. Studies analysing genome-wide changes in nucleosome levels have reported depletion of total histones at promoters of highly transcribed genes [18]. The amount of total H3 protein association was used as an indirect measure of nucleosome abundance [19]. When histone H3 protein content was analysed across the *ASNS* gene following AAR induction, there was a significant decrease in proximal promoter H3 levels, but no AAR-induced change at the -1 kb, + 1 kb or exon 7 regions (Figure 3A). Given that any change in histone modification has to be considered in the context of the total abundance of the substrate, the H3K4me3 data were normalized to histone H3 content. Normalization illustrated a significant increase in H3K4me3 at both the proximal promoter and + 1 kb regions of the *ASNS* gene (Figure 3A).

The H4Ac levels at the four regions of the gene were analysed. A significant increase in H4Ac levels at the *ASNS* proximal promoter and a trend toward higher H4Ac at the -1 kb and + 1 kb regions was observed (Figure 3B). In contrast, there was no signal obtained at exon 7, confirming that H4 acetylation is localized to the region near the *ASNS* promoter. The commercially available histone H4 antibodies were not as reliable as those for H3, so the H3 signal was used as an indicator of nucleosome association and the H4Ac amount was normalized to histone H3 abundance. These results illustrate that there is a preference for increased H4 acetylation at the proximal promoter (Figure 3B). The pan-H4Ac antibody can recognize acetylation at Lys⁵, Lys⁸, Lys¹² or Lys¹⁶. However, ChIP analysis with the antibody recognizing H4K8Ac revealed a signal that was greater than the non-specific IgG background (Figure 3C). When the H4K8Ac modification was analysed across the *ASNS* gene, the pattern of AAR-induced change obtained was similar to that for the pan-H4Ac antibody, suggesting that acetylation at H4K8 contributes significantly to the changes in H4Ac observed at the *ASNS* proximal promoter.

Rapid reversal of *ASNS* transcriptional activity upon removal of the AAR stress

Although the transcriptional events that occur during the induction phase of the AAR have been well described, much less has been reported about the changes in these processes following reversal of the amino acid stress. To further establish the molecular signals that control AAR signalling and stress-inducible transcription, a HisOH-washout protocol was used (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/449/bj4490219add.htm>). HepG2 cells were treated with HisOH for 4 h to increase *ASNS* transcriptional activity and then transferred to HisOH-free DMEM for 10 min to allow the HisOH to efflux out of the cells. After the 10 min washout, the cells were divided into two populations and incubated in either DMEM alone or DMEM containing HisOH for an additional 2 h. Surprisingly, the brief 10 min HisOH washout with DMEM caused an approximate 40 % reduction in *ASNS* transcriptional activity in cells assayed after a 30 min incubation, irrespective of whether they were incubated in the presence or absence of HisOH for that 30 min (Figure 4A). These results demonstrate that AAR-inducible *ASNS* transcription is rapidly reversed after removal of the amino acid stress. In cells cultured in DMEM for 2 h post-washout, *ASNS* transcriptional activity returned to basal levels, whereas in cells returned to DMEM +

HisOH for this 2 h, *ASNS* transcriptional activity was restored to the pre-washout, AAR-induced level (Figure 4A). To illustrate that this relatively rapid down-regulation of transcription rate was not related to the use of HisOH as an AAR trigger, a similar experiment was performed in cells cultured in complete MEM (minimal essential medium) or MEM lacking histidine (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/449/bj4490219add.htm>). As observed for the HisOH-based studies, within 30 min after adding histidine back to the medium of the previously histidine-deprived cells, the *ASNS* transcriptional activity was decreased by approximately a half, and after a 2 h incubation in complete MEM the activity was returned to the basal or 'fed' state.

To investigate these regulatory mechanisms for a second AAR-responsive gene, the *ATF3* gene was chosen. Like *ASNS*, *ATF3* is an ATF4-dependent amino-acid-responsive gene, but it also has mechanistic differences with the *ASNS* gene. For example, the transcriptional induction of the *ATF3* gene exhibits much greater dependence than *ASNS* on the HAT ATF2 [26], which is activated by the AAR through an ATF4-independent mechanism [34]. The possibility existed that a difference in which HAT activity contributes to the AAR mechanism for a particular gene may determine the kinetics of both transcriptional induction and reversibility of that induction. As shown in Figure 4(B), with regard to the reversibility following the HisOH washout, the *ATF3* gene behaved similarly to the *ASNS* gene. That is, after activation of the gene with a 4 h HisOH treatment, a 10 min incubation in DMEM lacking HisOH resulted in a decline in transcriptional activity, even when the cells were returned to the HisOH-containing medium for the remaining 30 min. Additionally, a complete return to basal levels after incubation in the absence of HisOH for 2 h was observed. To document the kinetics of AAR signalling during these washout studies, whole-cell protein extracts were probed by immunoblotting for p-eIF2 α and ATF4 protein content (Figure 4C). Consistent with the transcription data, HepG2 cells treated with HisOH for 4 h had increased levels of eIF2 α phosphorylation and ATF4 protein. In cells cultured for 2 h post-washout in DMEM, p-eIF2 α and ATF4 levels fully returned to the basal or 'fed' state ($t = 0$), indicating that AAR signalling was reversed, whereas cells returned to DMEM + HisOH for the 2 h post-washout period continued to express elevated amounts of p-eIF2 α and ATF4 protein (Figure 4C).

Histone changes at the *ASNS* and *ATF3* genes after removal of the AAR stress

To gain insight into the histone changes that occur during the reversal of AAR stress, ChIP analysis of the *ASNS* and *ATF3* genes were performed in HepG2 cells following the HisOH-washout protocol. To illustrate the mechanistic basis for the changes in transcriptional activity, the expected increase in RNA Pol II (Figure 5A) and ATF4 (Figure 5B) recruitment to the *ASNS* and *ATF3* promoters was observed in cells treated with HisOH for 4 h. After a HisOH washout in those cells incubated in DMEM for 2 h, both Pol II and ATF4 binding reverted to the basal level in a manner that paralleled the decline in transcription (Figure 4). As a negative control, it was documented that binding of non-specific IgG antibody to both the *ASNS* and *ATF3* genes was negligible at all time points tested (Figure 5C). Consistent with the results shown in Figure 3, AAR induction with HisOH for 4 h caused a significant decrease in total histone H3 levels associated with the promoters of both *ASNS* and *ATF3* (Figure 6A), suggesting that nucleosome remodelling is occurring at these genes. When the cells were subjected to HisOH washout and then cultured in DMEM alone for 2 h, the H3 levels did not return to the DMEM control levels (0 or 4 h DMEM values), but remained slightly higher than those cells transferred to DMEM + HisOH, which continued declining relative to the level observed at 4 h of HisOH treatment (Figure 6A). These results indicate that, despite the nearly complete reversal of the transcription activity within 2 h after transfer of the HisOH-treated cells back to DMEM alone (Figure 4), the nucleosome remodelling that occurred at the *ASNS* and *ATF3*

promoters during HisOH treatment was not as rapidly reversed. To assess whether or not this response was also true for specific histone marks, the changes in histone pan-H4 acetylation were assessed at both the *ASNS* and *ATF3* promoters. Although the absolute values obtained for H4Ac levels increased relatively modestly in response to the AAR (Figure 6B), normalization of the data for the reduction in total histone present illustrated a significant effect on acetylation (Figure 6C). Reversal of the AAR by HisOH washout and a subsequent 2 h incubation in DMEM alone resulted in a complete return of the H4Ac levels back to control DMEM values, whereas continued incubation in HisOH-containing medium caused a further increase.

For both *ASNS* and *ATF3*, the absolute level of H3K4me3 was unchanged in the proximal promoter region (Figure 7A, left-hand panel) and significantly increased at the + 1 kb region (Figure 7B, left-hand panel). After normalizing the H3K4me3 data for the abundance of H3 protein, the H3K4me3 level for cells maintained in HisOH for the 2 h post-washout period continued to rise, whereas in those cells that were transferred to the DMEM alone, the AAR-induced H3K4me3 levels at both the proximal promoter and the + 1 kb regions remained relatively constant and did not return to the basal level (Figure 7, right-hand panels). These results for H3K4me3 are in contrast with the relatively rapid and complete reversal of the Pol II, ATF4 and H4Ac associations. However, these results are similar to an observation for the galactose-dependent induction of the yeast *GAL10* promoter [35] in which H3K4me2 and H3K4me3 modifications remained elevated after removal of galactose and only returned to basal levels after 5 h, despite the reversal of the elevated RNA Pol II binding and Set1 H3K4-methyltransferase association back to basal levels within 20 min. The authors of that study [35] proposed that the more persistent H3K4me3 modification functioned as a memory mark for transcriptional activity and they proposed that this mark alters the sensitivity of the gene to further activation, as shown later for *GAL1* by several independent studies discussed below.

Re-induction of *ASNS* transcription in response to a second round of AAR stress

The possibility was explored that the persistence of the promoter H3 loss and the enhanced H3K4me3 at + 1 kb may alter the kinetics of transcriptional activation if the AAR were induced a second time. Using the *ASNS* gene as the reporter, after reversing the initial 4 h AAR-induced activity with a 2 h incubation in DMEM lacking HisOH, one-half of the cells were then transferred back to HisOH-containing medium to trigger a second round of activation (Figure 8). The time course and magnitude of transcriptional activation during the first and second round of HisOH treatment were nearly superimposable, indicating that the 2 h reversal period does not render the gene resistant to, or primed for, re-activation. These data suggest that the slow reversibility of the H3 abundance or the presence of the H3K4me3 mark does not alter AAR responsiveness of the *ASNS* gene.

DISCUSSION

Using the *ASNS* and *ATF3* genes as examples, the results of the present study provide several novel observations that expand the current comprehension of transcriptional regulation during amino acid deprivation. (i) There are temporal and spatial distribution shifts of H4K8Ac and H3K4me3 histone modifications, marks that are indicative of gene activation. (ii) A decline in total histone H3 association at the promoter region of these two genes provides evidence for nucleosome remodelling during AAR induction. (iii) The data reveal that just 10 min after removal of the signal that triggers the AAR, a significant decrease in transcriptional activity is apparent and, within 2 h, the induction of these genes is completely reversed. (iv) After AAR-stress reversal, ATF4 recruitment, as well as the H4Ac modification, rapidly decline in parallel with the reduction in transcription. In contrast, both the decline in total H3 association at the promoter and the increase in H3K4me3

modification downstream of the TSS persist during the 2 h reversal phase, but the retention of these changes does not enhance or suppress the response of the gene to a subsequent round of activation of the AAR.

Collectively, the results from the present study confirm that in the 'basal' state there is a low-level occupancy of ATF4 at the CARE and of RNA Pol II at the proximal promoter of amino-acid-responsive genes. The chromatin in the region of the promoter is characterized by the presence of H4Ac and H3K4me3 histone modifications and an absence of H3K27me3 modification, indicating a permissive state for transcription. These observations are consistent with the physiological data indicating that most amino-acid-responsive genes are typically not in an 'off' state, but rather exhibit a constant low level of expression. Following AAR induction, within 45 min there is a significant increase in recruitment of ATF4, which is accompanied by nucleosome remodelling, as shown by loss of histone H3 protein association and enhancement of H4Ac and H3K4me3 modifications. These changes represent the 'activated' state and occur in parallel with increased Pol II binding and transcription. Interestingly, we have shown that ectopic expression of ATF4 alone, in the absence of activating the AAR pathways, is sufficient to trigger transcription from the *ASNS* gene, a response that is also associated with enhanced histone H4 acetylation [36]. Additionally, preliminary experiments suggest that the elevating ATF4 protein alone leads to an increase in H3K4me3 at the *ASNS* promoter (J. Shan and M.S. Kilberg, unpublished work). This direct action by ATF4 is consistent with the proposal that the enzymes catalysing histone modifications are targeted to regulatory regions by enhancer-binding transcription factors, and that the histone modifications correlate with active transcription because they affect the physical properties of nucleosomes and maintain active chromatin [37].

Using a HisOH-washout protocol provided the ability to study the ramifications of rapidly reversing the transcriptional activation, a process that has not been systematically investigated for amino-acid-responsive genes. Within 2 h following removal of the AAR stress, the *ASNS* and *ATF3* genes revert to the basal state, as illustrated by a decrease in ATF4 and Pol II binding and a reversal of the increase in H4Ac. Interestingly, the AAR-induced decline in nucleosome abundance, as estimated by H3 protein association, and the increase in H3K4me3 levels still persist at 2 h after removal of HisOH. Although these two modifications may represent molecular memory of the recent stress-induced transcription from the gene, the time course and magnitude of *ASNS* induction after a second round of HisOH treatment closely mirrors that of the initial activation. This result indicates that the cells do not enter a long refractory period, nor are they primed for an enhanced response. The rapid reversal of transcription following removal of the amino acid stress also functions in the *in vivo* setting. Carraro et al. [28] showed that *Drosophila TRB3* transcription was increased in the liver of mice fed a leucine-deficient diet and that this up-regulation was quickly reversed when these mice were re-fed a complete diet for 2 h. Also, after re-feeding the leucine-replete diet there was a loss of AAR-induced ATF4 recruitment to the *TRB3* genomic CARE region. The present study provides evidence for a similar effect at the *ASNS* and *ATF3* genes, but this cell-culture model allows for study of shorter times and revealed that even a 10 min removal of the AAR stress caused a rapid reduction in transcriptional activity. An earlier study from our group used a Tet (tetracycline)-inducible ATF4 expression system to establish that although ATF4 in the absence of other AAR signals triggered increased *ASNS* transcription, a greater amount of ATF4 protein was required to achieve the same degree of *ASNS* transcriptional induction triggered by the AAR [36]. Moreover, the observation that the Tet-induced ATF4 occupancy at the *ASNS* promoter caused a smaller increase in histone acetylation compared with that following AAR induction suggests that the associated histone modifications are perhaps influenced by activation of other AAR signalling pathways, such as the MAPK (mitogen-activated protein

kinase) cascades and their downstream effectors [38]. A future study using the Tet-inducible ATF4 expression system to examine the reversal of the AAR in the presence or absence of ectopic ATF4 protein will aid in delineating the ATF4-dependent and the ATF4-independent changes in histone modifications at *ASNS* and other CARE-containing genes.

Beyond amino acid availability, other macronutrients also trigger acute changes in co-transcriptional histone modifications. For example, activation of transcription from the low-density lipoprotein receptor and hydroxymethyl glutaryl CoA reductase promoters by the lipid-sensing SREBP (sterol-regulatory-element-binding protein) is associated with an increase in H3 acetylation [39]. Likewise, in parallel with a glucose-induced recruitment of the ChREBP (carbohydrate-response-element-binding protein), Shalev and colleagues observed increased histone H4 acetylation, but not histone H3 acetylation or H3K4 trimethylation, at the thioredoxin-interacting protein promoter [40]. Conversely, Burke et al. [41] observed that glucose-enhanced ChREBP binding to the L-type pyruvate kinase gene caused both H3 and H4 acetylation at the promoter, as well as increased H3K4me3 within the coding region. Antagonism of the glucose-mediated expression of the pyruvate kinase gene by cAMP was associated with decreased H3 and H4 acetylation, decreased H3K4me3 abundance, and increased methylation of H3K9 in the coding region [41]. In yeast, several groups have observed that, following a previous instance of galactose-induced expression, reinduction of *GAL1* transcription occurs with faster kinetics and a number of separate mechanisms have been proposed to explain this positive memory effect [42–45]. Conversely, Zhou and Zhou [46] examined the transient H3K4 di- and tri-methylation at the *GAL1* gene and reported that H3K4 methylation actually inhibited re-induction of *GAL1*, because binding of the ISWI (Imitation Switch) nucleosome-remodelling complex to the *GAL1* gene led to the localization of Pol II predominantly to the 3' region, preventing *GAL1* re-induction. In contrast with these yeast *GAL1* studies, the slow reversal after refeeding of the total nucleosome content and the H3K4me3 mark near the mammalian *ASNS* promoter does not appear to have a significant impact on the kinetics of *ASNS* transcription during a second round of AAR stress. The relatively long stability of the *ASNS* mature mRNA during the AAR may mean that the cell does not need to alter the *ASNS* transcriptional kinetics during successive periods of activation [12]. However, the observation that there is a significant reduction in histone H3 association at the *ASNS* and *ATF3* promoters suggests that nucleosome remodelling enzymes act on both of these ATF4-responsive genes. In yeast, Hinnebusch and colleagues have observed that GCN4, the functional counterpart of ATF4, interacts with the SWI/SNF nucleosome remodelling complex [47]. Indeed, those authors showed that the interaction between GCN4 and SWI/SNF was important for GCN4-mediated transcription from the *HIS3* gene. Additionally, it was observed that GCN4 recruits other chromatin-modifying co-activators, but the subset of these co-activators recruited was gene specific [48].

A future challenge in characterizing the epigenetic signals that contribute to amino-acid-dependent transcription is to identify the enzyme writers, readers and erasers of the observed histone modifications. The H3K4me3 mark is catalysed in yeast by the Set1 methylase as part of a Set1 (COMPASS) methyltransferase complex [49]. There are more than six Set1-like mammalian homologues, which are divided into two main classes of histone H3K4me3 methylases, the Set proteins hSet1A and hSet1B, and the MLL (mixed-lineage leukaemia) (MLL1–4) proteins, which are components of COMPASS-like complexes [49]. Consistent with the changes in the H3K4me3 mark described in the present paper, we have shown that the mRNA expression of several members of the MLL family is increased by the AAR (MLL2, 3.4-fold; MLL3, 2.9-fold; and MLL4, 2.3-fold) [8]. At certain genes, the H3K4me3 mark has been observed to aid in the recruitment of the basal transcription factor TFIID via TAF3 [50], and subsequently cause transcription initiation through recruitment of the basal transcription machinery [51]. Therefore our observation of AAR-dependent induction of

several MLL genes suggests that an increase in the H3K4me3 mark may be a critical event for amino-acid-responsive gene transcription initiation and maintenance [8].

Although Fafournoux and colleagues [26] have observed that p-ATF2 functions as a HAT during the AAR induction of the *ATF3* and *CHOP* genes, the induction of *ASNS* during the AAR is much less dependent on ATF2 action [26,52]. Furthermore, amino acid deprivation causes increased association of another HAT, PCAF [p300/CREB (cAMP-response-element-binding protein)-binding protein-associated factor], with the *CHOP* gene, but not with the *ASNS* gene [12,53]. Therefore other HAT complexes must be responsible for the AAR-induced increase in histone acetylation at the *ASNS* locus. We have preliminary data documenting that p300 is bound to the *ASNS* promoter in the basal or 'fed' condition and that there is a 2-fold increase in bound p300 during the AAR; however, activation of the *ASNS* gene by the AAR occurs in p300-deficient MEF (mouse embryonic fibroblast) cells, suggesting that redundancy for its action may occur (results not shown). Conversely, increased histone acetylation may also result from decreased HDAC (histone deacetylase) function [54]. Cherasse et al. [55] observed that the low level of *CHOP* transcription in amino-acid-replete cells was characterized by HDAC3 binding in the region of the *CHOP* CARE sequence in the presence of leucine, and that this HDAC3 binding was diminished by leucine deprivation. Treatment of cells with the HDAC inhibitor TSA (trichostatin A) de-repressed *CHOP* expression, suggesting that a dynamic amino-acid-dependent HDAC3 association with the gene may be a component of transcriptional control during the AAR [55]. Future experimentation will be necessary to firmly establish the AAR-associated HAT and HDAC activities.

Transcriptional regulation by nutrient availability is a recognized mechanism for control of a wide range of cellular processes. Rapid and reversible changes in nutrient signalling and transcriptional control by enhancer-binding proteins is beginning to be characterized for several model systems, including amino-acid-responsive genes. Far less is known about the impact of nutrients on epigenetic changes within the genome. The initial studies suggest that highly reversible histone modifications occur within minutes of changes in amino acid supply. The mechanisms and physiological consequences of these epigenetic modifications will require further investigation, as will the identification of the corresponding enzymes responsible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AAR	amino acid response
ASNS	asparagine synthetase

ATF	activating transcription factor
CARE	C/EBP–ATF response element
C/EBP	CCAAT/enhancer-binding protein
ChIP	chromatin immunoprecipitation
CHOP	C/EBP homology protein
ChREBP	carbohydrate-response-element-binding protein
DMEM	Dulbecco's modified Eagle's medium
eIF2	eukaryotic translation initiation factor 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCN	general control non-derepressible
HAT	histone acetyltransferase
HDAC	histone deacetylase
HisOH	histidinol
MEM	minimal essential medium
MLL	mixed-lineage leukaemia
p-	phospho-
Pol II	RNA polymerase II
qPCR	quantitative PCR
SFRP1	secreted frizzled-related protein 1
SNAT2	sodium-dependent neutral amino acid transporter 2
TAF	TATA-binding protein-associated factor
Tet	tetracycline
TRB3	tribbles homologue 3
TSS	transcription start site

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Figure 1. Identification of AAR-inducible histone modifications at the *ASNS* gene
(A) Location of the amino-acid-responsive ATF4-binding CARE site. Arrows show the primer pairs used to scan the gene by ChIP analysis. **(B)** HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for 4 h and then the binding of ATF4 and the relative abundance of histone modifications (H4Ac and H3K36me3) at the promoter and exon 7 within the *ASNS* gene were analysed. **(C)** The H3K27me3 modification level at the proximal promoter regions of the *ASNS* and *SFRP1* genes was analysed by ChIP in HepG2 cells cultured in DMEM $\pm$ 2 mM HisOH for 16 h. Within each ChIP experiment, a non-specific IgG was used as a negative control. Values, plotted as the ratio to input DNA, are means $\pm$ S.D. for at least three samples. * P 0.05 relative to the DMEM control.

Figure 2. Temporal analysis of transcriptional induction and H3K4me3 levels at the *ASNS* gene during the AAR

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for 0–24 h. **(A)** Total RNA was isolated at the indicated times and qPCR was performed to analyse the *ASNS* transcriptional activity (top panel) and steady-state mRNA levels of *ASNS* and *GAPDH* (bottom panel). The data for *ASNS* mRNA is normalized relative to the DMEM value at zero time. **(B)** ChIP analysis was performed to analyse the H3K4me3 levels at the *ASNS* proximal promoter and at + 1 kb downstream of the TSS (see Figure 1A for primer locations and Supplementary Table S1 at <http://www.BiochemJ.org/bj/449/bj4490219add.htm> for primer sequences). The data are graphed as the fold-change for the ratio to input DNA and are normalized to the H3K4me3 value for the 2 h DMEM sample. Values are means $\pm$ S.D. for at least three samples. * P < 0.05 relative to the DMEM control at the same time period.

Figure 3. Analysis of histone modifications during the AAR

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for 4 h. The levels of histone modifications for H3K4me3 (A), pan-H4Ac (B) and H4K8Ac (C) were analysed along with total histone H3 abundance by ChIP at the *ASNS* gene regions indicated (see Figure 1A for primer locations and Supplementary Table S1 at <http://www.BiochemJ.org/bj/449/bj4490219add.htm> for primer sequences). The data are plotted as the ratio to input DNA and, where indicated, the values for the histone modifications were normalized to the signal obtained for total histone H3 at the respective regions (bottom graph in each panel labelled 'Normalized signal'). Values are means $\pm$ S.D. for at least three samples. * P < 0.05 relative to the DMEM control.

Figure 4. Reversal of transcription after removal of the AAR stress

A HisOH-washout protocol was used to test the effect of reversing the AAR stress by removing the HisOH for a 10 min period and then transferring the cells to DMEM ± HisOH (see additional details in Supplementary Figure S1 at <http://www.BiochemJ.org/bj/449/bj4490219add.htm> and the main text). Total RNA was isolated at the indicated times and qPCR was performed to analyse the *ASNS* (A) or *ATF3* (B) transcriptional activity. The data for transcriptional activity were calculated as the ratio to the *GAPDH* mRNA control and then plotted relative to the DMEM value at zero time. Values are means ± S.D. for at least three samples and, where not shown, the error bars are contained within the symbol. * P

0.05 relative to the DMEM control for that time point. The hash mark following the 4 h HisOH data point indicates the 10 min period of HisOH washout. (C) Whole-cell protein extracts were collected after a 4 h initial incubation in DMEM ± HisOH and then at 0.5, 1 or 2 h after the 10 min HisOH washout. The indicated protein levels were analysed by immunoblotting using β-actin to monitor loading. The immunoblot shown is representative of multiple experiments.

Figure 5. Promoter-associated proteins after reversal of the AAR stress

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for the indicated times. ChIP analysis of the *ASNS* and *ATF3* genes was performed to analyse promoter binding of RNA Pol II (A) or ATF4 (B). A non-specific IgG was used as a negative control (C). The data are plotted as the ratio to input DNA. The hash mark following the 4 h HisOH data point indicates the 10 min period of HisOH washout. Values are means $\pm$ S.D. for at least three samples and, where not shown, the error bars are contained within the symbol. * P < 0.05 relative to the DMEM control for the same time point.

Figure 6. Acetylation of histone H4 after reversal of the AAR Stress

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for the indicated times. ChIP analysis of the *ASNS* and *ATF3* genes was performed to analyse association of total histone H3 (A) or pan-H4Ac (B). In (C), the ratio of H4Ac/total H3 is illustrated as the 'Normalized signal' for both genes. The data are plotted as the ratio to input DNA. The hash mark following the 4 h HisOH data point indicates the 10 min period of HisOH washout. Values are means $\pm$ S.D. for at least three samples and, where not shown, the error bars are contained within the symbol. * P < 0.05 relative to the DMEM control for the same time point.

Figure 7. H3K4 trimethylation after reversal of the AAR stress

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for the indicated times. ChIP analysis of the *ASNS* and *ATF3* genes was performed to analyse association of histone H3K4me3 at both the promoter region (**A**) and at the + 1 kb region (**B**). The absolute abundance of the H3K4me3 mark is illustrated as the ratio to input DNA (left-hand panels) and in the right-hand panels, the values after normalization for nucleosome abundance are shown as the ratio of H3K4me3/total H3. The hash mark following the 4 h HisOH data point indicates the 10 min period of HisOH washout. Values are means $\pm$ S.D. for at least three samples and, where not shown, the error bars are contained within the symbol. * P < 0.05 relative to the DMEM control for the same time point.

Figure 8. Analysis of *ASNS* transcriptional activity in response to a second round of AAR activation

HepG2 cells were cultured in DMEM $\pm$ 2 mM HisOH for 0–4 h to activate the AAR. A batch of cells incubated with HisOH for 4 h was then transferred to DMEM lacking HisOH for 2 h to reverse the AAR induction. After a 2 h period in DMEM alone, one-half of the cells were transferred to DMEM + 2 mM HisOH for an additional 4 h to provide a second round of AAR activation. These changes in culture medium are summarized below the data using broken lines to designate DMEM and a solid lines to designate DMEM + HisOH. At the indicated time points, total RNA was isolated and qPCR was performed to analyse the *ASNS* transcriptional activity and steady-state mRNA levels of *GAPDH*. The data for *ASNS* transcriptional activity were calculated as the ratio to the *GAPDH* control and then plotted relative to the DMEM value at zero time. Values are means $\pm$ S.D. for at least three samples and, where not shown, the error bars are contained within the symbol. * P < 0.05 relative to the DMEM control for the same time point.