Characterization of the amino acid response element within the human sodium-coupled neutral amino acid transporter 2 (SNAT2) System A transporter gene

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The neutral amino acid transport activity, System A, is enhanced by amino acid limitation of mammalian cells. Of the three gene products that encode System A activity, the one that exhibits this regulation is SNAT2 (sodium-coupled neutral amino acid transporter 2). Fibroblasts that are deficient in the amino acid response pathway exhibited little or no induction of SNAT2 mRNA. Synthesis of SNAT2 mRNA increased within 1–2 h after amino acid removal from HepG2 human hepatoma cells. The amino acid responsive SNAT2 genomic element that mediates the regulation has been localized to the first intron. Increased binding of selected members of the ATF (activating transcription factor) and C/EBP (CCAAT/enhancer-binding protein) families to the intronic enhancer was established both *in vitro* and *in vivo*. In con-

trast, there was no significant association of these factors with the SNAT2 promoter. Expression of exogenous individual ATF and C/EBP proteins documented that specific family members are associated with either activation or repression of SNAT2 transcription. Chromatin immunoprecipitation analysis established *in vivo* that amino acid deprivation led to increased RNA polymerase II recruitment to the SNAT2 promoter.

Key words: activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein (C/EBP), eukaryotic initiation factor 2 α (eIF2 α), general control non-derepressible-2 (GCN2), nutrient starvation, sodium-coupled neutral amino acid transporter 2 (SNAT2).

INTRODUCTION

The mammalian System A neutral amino acid transporter activity is a sodium-dependent, secondary active transporter that is expressed in all nucleated mammalian cells [1–4]. The level of System A activity is usually quite low in cells that are slowly dividing or in metabolic homeostasis. However, System A transport activity is up-regulated in response to amino acid deprivation [5,6]. The cDNA sequences for three System A-encoding isoforms have been cloned, and the nomenclature SNAT1 (sodium-coupled neutral amino acid transporter 1), SNAT2 and SNAT4 was adopted [7]. It has been documented that SNAT2 is the primary isoform induced when mammalian cells are deprived of amino acids, as illustrated by the direct correlation between SNAT2 mRNA expression and System A transport activity [8,9].

The signalling pathway that is triggered in response to amino acid deprivation is referred to as the AAR (amino acid response), but the molecular mechanisms that detect protein/amino acid deficiency, mediate the AAR signal transduction cascade and eventually initiate increased transcription are still not completely understood. Following amino acid depletion, the GCN2 (general control non-derepressible-2) kinase is activated and phosphorylates the translation initiation factor eIF2 α (eukaryotic initiation factor 2 α), which, in turn, decreases global protein synthesis. However, phosphorylation of eIF2 α actually favours increased translation of a selected number of mRNAs, including that coding for ATF4 (activating transcription factor 4) [10]. Thus one of the mechanisms by which cells respond to amino acid stress is

by increasing transcription from a subset of ATF4 target genes. Among these genes are those that contain AAREs (AAR elements) that mediate the enhanced transcription (reviewed in [11]), and function as enhancer elements [12,13]. AARE-binding proteins have only been reported for two genes, ASNS (asparagine synthetase) and CHOP [C/EBP (CCAAT/enhancer-binding protein) homology protein]. Both of these AARE sites have a 9–10 bp core element, but the sequences differ by 2 nt between the genes [14]. Although both AARE sites bind ATF4 [15–17], the CHOP sequence also binds ATF2 [16], whereas the ASNS site does not [15,17]. ChIP (chromatin immunoprecipitation) analysis revealed that within 30–45 min after removal of a single amino acid from the culture medium, ATF4 binding to the ASNS promoter was increased, and this increase continued over the next 4 h [17]. During a more extended amino acid limitation period (4–24 h), the ASNS promoter binding of ATF3 [17–19] and C/EBP β [17,20] is increased at a time when the transcription activity is declining [17]. As further evidence for activation by ATF4, the activity of ASNS promoter-driven transcription was induced in ATF4-overexpressing cells [15], and expression of a dominant-negative ATF4 mutant prevented starvation induction of the ASNS promoter. However, whether or not these transcription factors regulate the SNAT2 gene has not been investigated previously.

The SNAT2 gene represents an important mechanistic model to investigate how changes in nutrient-regulated transcription are triggered and maintained. From a physiological viewpoint, understanding the regulation of System A-mediated plasma membrane

Abbreviations used: AAR, amino acid response; AARE, AAR element; ASNS, asparagine synthetase; ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homology protein; CREB, cAMP-response-element-binding protein; eIF2 α , eukaryotic initiation factor 2 α ; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; FL, full length; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCN2, general control non-derepressible-2; GST, glutathione S-transferase; hnRNA, heterogeneous nuclear RNA; LAP, liver-enriched activating protein; LIP, liver-enriched inhibitory protein; MEF, mouse embryonic fibroblast; MEM, minimal essential medium; Pol II, polymerase II; RT, reverse transcriptase; SNAT2, sodium-coupled neutral amino acid transporter 2; SV40, simian virus 40; TRB3, tribbles-related protein 3.

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transport is fundamental to understanding how changes in plasma amino acid levels impact upon cellular metabolism. We have characterized amino acid-regulated transcription from a human SNAT2 genomic fragment [21] and, subsequently, identified an AARE [13]. Interestingly, the genomic location of the SNAT2 AARE differs from that in either the ASNS or the CHOP promoter in that it is located within the first intron [13]. The 9 bp core sequence of the SNAT2 AARE is identical with that in the CHOP promoter, and consequently, differs by 2 nt from the ASNS AARE [12,22]. Another difference is that the SNAT2 AARE activity is influenced by a highly conserved CAAT box located 11 bp upstream, which is not absolutely required for the amino acid-dependent response, but contributes to its absolute magnitude [13].

The purpose of the present study was to identify the transcription factors that bind to the intronic SNAT2 AARE following amino acid limitation. Consistent with the current understanding of the AAR pathway, SNAT2 mRNA accumulation following amino acid limitation was blocked in MEFs (mouse embryonic fibroblasts) deficient for GCN2 or expressing a mutated eIF2 α (S51A). The results show that the SNAT2 AARE forms specific protein complexes when incubated with nuclear protein extracts *in vitro* and that the abundance of these complexes is increased when extracts from amino acid-deprived cells are tested. By antibody supershift analysis *in vitro*, evidence was obtained for increased AARE binding of ATF4, C/EBP α , C/EBP β and C/EBP δ following amino acid deprivation. Overexpression studies provided further evidence for a role of ATF and C/EBP proteins in the regulation of SNAT2 transcription, and following amino acid depletion, ChIP analysis documented the kinetics of transcription factor binding to the intronic AARE *in vivo*.

MATERIALS AND METHODS

Cell culture

HepG2 human hepatoma cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The medium used was modified Eagle's MEM (minimal essential medium; pH 7.4) (Mediatech, Herndon, VA, U.S.A.), supplemented with 1× non-essential amino acids, 4 mM glutamine, 100 µg/ml streptomycin sulphate, 100 units/ml penicillin G, 0.25 µg/ml amphotericin B and 10% (v/v) FBS (fetal bovine serum). Cells were replenished with fresh MEM and serum, 12 h before all experiments, to ensure that little or no nutrient deprivation occurred prior to initiating amino acid removal. For amino acid deprivation experiments, cells were incubated for a specific time period in either complete MEM or MEM lacking histidine, each containing 10% dialysed FBS.

Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.): ATF2, sc-187 (C-terminal); ATF2, sc-242 (basic region); ATF3, sc-188; ATF4 [CREB-2 (cAMP-response-element-binding protein-2)], sc-200; C/EBP α , sc-61; C/EBP β , sc-150; C/EBP δ , sc-636; C/EBP ϵ , sc-158; RNA Pol II (polymerase II), sc-899; and normal rabbit IgG, sc-2027.

Plasmids

SNAT2-driven luciferase reporter constructs were described previously [13]. The pGL3 and pGL3 promoter vectors were obtained from Promega (Madison, WI, U.S.A.). The rat wild-type and dominant-negative ATF4 cDNAs, generously provided by Dr Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA, U.S.A.) [23], were cloned into the pcDNA3.1/Myc-His(−) vector

(Invitrogen), and expression was driven by the cytomegalovirus promoter. ATF3 expression plasmids were described previously [18]. A cDNA for the complete ATF2 coding region was obtained by RT (reverse transcriptase)–PCR using the primers: sense, 5'-ATGAAATTCAGTTACATGTGA-3' and antisense, 5'-CAAGGAAAGACCAGTTTC-3'. The purified ATF2 PCR product was cloned into the pcDNA3.1 vector at the BamHI site and its integrity was verified by sequencing. The pSCT vector containing either the activating isoform of rat C/EBP β [LAP (liver-enriched activating protein)] or the dominant-negative isoform [LIP (liver-enriched inhibitory protein)] of C/EBP β , originally described by Descombes and Schibler [24], was kindly provided by Dr Harry S. Nick (University of Florida, Gainesville, FL, U.S.A.). The murine C/EBP α expression plasmid was provided by Dr Peter Johnson (The National Cancer Institute, Bethesda, MD, U.S.A.) and the C/EBP δ expression plasmid was obtained from Dr Steven McKnight (University of Texas, Southwestern, Dallas, TX, U.S.A.).

MEF cell lines

MEF cells deficient for GCN2 [10] or expressing a non-activatable eIF2 α (S51A) [25] were generated by the laboratories of Dr David Ron at NYU School of Medicine (New York, NY, U.S.A.) and Dr Randal Kaufman at the University of Michigan respectively.

Transient transfection and luciferase assays

Human HepG2 hepatoma cells were seeded in 24-well plates at a density of 1.2×10^5 cells/well, supplied with complete MEM and grown for 24 h. Transfection was performed with 1 µg of firefly luciferase reporter plasmid DNA per 1.2×10^5 cells using Superfect reagent (Qiagen, Valencia, CA, U.S.A.) at a ratio of 1 µg of DNA/6 µl of reagent, according to the manufacturer's instructions. Each well also received 10 ng of pRL-SV40 (where SV40 is simian virus 40) plasmid (*Renilla* luciferase) to serve as a control for transfection efficiency. The amount of co-transfected transcription factor expression plasmid was 100 ng/well, and the total amount of transfected DNA was kept constant among experimental groups by the addition of empty pcDNA3.1 plasmid. After 3 h, cells were rinsed once with PBS and given fresh MEM. At 16 h post-transfection, the medium was removed, the cells were rinsed once with PBS and incubated for 10 h in 1 ml/well of either complete MEM or MEM lacking histidine (MEM – His), each supplemented with 10% dialysed FBS. After the completion of treatment, the cells were rinsed with PBS, lysed with 100 µl of 1× Passive lysis buffer (Promega) and then subjected to one freeze–thaw cycle to ensure complete disruption of the membranes. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay system (Promega). Replicates of six transfections were performed for each experimental condition, and all experiments were repeated with separate batches of cells to ensure reproducibility of results.

Nuclear extract preparation and EMSA (electrophoretic mobility-shift assay)

HepG2 cells were seeded on 150 mm dishes at a density of 15×10^6 cells per dish. After 16 h of culture, the cells were washed twice with PBS and incubated in either complete MEM or MEM lacking histidine, both supplemented with 10% dialysed FBS. The nuclear extraction was performed as previously described [20]. Protein concentration was determined using a modified Lowry assay [26]. Single-stranded oligonucleotides were annealed by adding 0.4 nmol of each, with 10 µl of 10× annealing buffer (100 mM Tris/HCl, pH 7.5, 1 M NaCl and 10 mM EDTA) in a total volume of 100 µl. The oligonucleotide solution was

Table 1 EMSA probes and competitor oligonucleotides

Those nucleotides that are boldfaced and underlined are the mutated ones from the wild-type sequences given just above them. AP-1, activating protein-1; WT, wild-type.

Name	Oligonucleotide sequence
WT SNAT2	5'-TTGACAATGCACGATCGATATTGCATCAGTTTCTTT-3'
CAAT + AARE	5'-AACTGTACGTGCTAGCTATAACGTAGTCAAAAGAA-3'
WT SNAT2 AARE 21-mer	5'-ATCGATATTGCATCAGTTTCTAGCTATAACGTAGTCAAAAG-3'
Mut SNAT2 AARE 21-mer	5'-ATCGATAATG CC CAGTTTCTAGCTAT ACGG GTCAAAAG-3'
CHOP AARE	5'-TTGCCAAACATTGCATCATCCCGC-3'
	5'-AACGGTTTGTAACTAGTAGGGGCG-3'
ASNS AARE	5'-CCTCGCAGGCATGATGAACTTCCCGC-3'
	5'-GGAGCGTCCGTACTCTTGAAGGGCG-3'
WT SNAT2	5'-TTGGGAACATTGACAATCGACGATCGA-3'
CAAT 28-mer	5'-AACCTGTAACTGTTAGCTGCTAGCT-3'
Mut SNAT2	5'-TTGGGAACATTGAC CTGC GACGATCGA-3'
CAAT 28-mer	5'-AACCTGTAACT GGAC GCTGCTAGCT-3'
C/EBP consensus	5'-TGCAGATTGCGCAATCTGCA-3'
	5'-ACGTCTAACGCTTAGACGT-3'
AP-1 consensus	5'-CGCTTGATGACTCAGCCGAA-3'
	5'-GCGAACTACTGAGTCGGCCTT-3'
CREB/ATF consensus	5'-AGAGATTGCCTGACGTGAGAGCTAG-3'
	5'-TCTCTAACGGACTGCACTCTCGATC-3'
Unrelated	5'-GCTTATCGATACCGTCGACCTCGAGATCT-3'
	5'-CGAATAGCTATGGCAGCTGGAGCTCTAGA-3'

heated to 95 °C for 5 min and then allowed to cool gradually to 4 °C over 2 h. The oligonucleotides used as either EMSA probes or unlabelled competitors are listed in Table 1. The double-stranded oligonucleotides were radiolabelled by extension of overlapping ends with Klenow fragment in the presence of [α -³²P]dATP. For each binding reaction, 10 μ g of nuclear extract protein was incubated with 40 mM Tris base (pH 7.5), 200 mM NaCl, 2 mM dithiothreitol, 10 % (v/v) glycerol, 0.05 % (v/v) Nonidet P40, 3 μ g of poly(dI-dC)·(dI-dC) (Amersham Biosciences, Piscataway, NJ, U.S.A.), 0.04 pmol of unrelated DNA and 0.05 mM EDTA for 20 min on ice. The radiolabelled probe was added at a concentration of 0.02 pmol/reaction (~20000 c.p.m.), and unlabelled competitor oligonucleotides were added at the indicated concentrations. The reaction mixture, 20 μ l final volume, was incubated at room temperature (22 °C) for 20 min. If an antibody was tested for supershift, it was added and a second 20 min incubation was included. The reactions were subjected to electrophoresis as described previously [20].

Expression and purification of GST (glutathione S-transferase)-ATF4 fusion protein

A fragment of the cDNA (nt +883 to +1938) coding for full length of human ATF4 (GenBank® accession number NM.001675) was cloned in-frame with GST into the EcoRI site of pGEX-6-P (Amersham Biosciences). The resulting vector was named pGEX-ATF4 and the fusion protein expressed from this vector was named GST-ATF4, which was introduced into *Escherichia coli* BL21(DE3) following standard methods. For large-scale GST-ATF4 production, the cells were inoculated in a 1 litre culture and incubated at 37 °C until the absorbance (A) detected at 600 nm reached 1.2. Protein expression was induced by adding 0.05 mM isopropyl β -D-thiogalactoside and grown for 4 h at 30 °C. The cells were lysed and GST-ATF4 was purified with glutathione-agarose affinity chromatography (Sigma, St. Louis,

MO, U.S.A.). The purified ATF4 concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, U.S.A.) [26a].

Transcription activity and steady-state mRNA determination

Total RNA was isolated from HepG2 cells using the Qiagen RNeasy kit (Qiagen) and including DNase I treatment before final elution to eliminate any DNA contamination. To measure the transcriptional activity from the SNAT2 gene, oligonucleotides derived from SNAT2 exon 4 and intron 4 were used to measure the short-lived unspliced transcript [hnRNA (heterogeneous nuclear RNA)]. This procedure for measuring transcriptional activity is based on that described by Lipson and Baserga [27], except that we analysed hnRNA levels by quantitative real-time PCR using the DNA Engine Opticon 2 system (MJ Research, Reno, NV, U.S.A.) and SYBR Green. Reactions without RT were performed as a negative control to rule out amplification from any residual genomic DNA. These tests were always negative. The primers for amplification were: sense primer, 5'-GCAGTGGAAATCCTTGGGCTTTC-3', and antisense primer, 5'-CCCTGCATGGCAGACTCACTACTTA-3'. The reaction mixtures were incubated at 63 °C for 30 min followed by 95 °C for 15 min to activate the Taq polymerase and amplification of 35 cycles of 95 °C for 15 s, and 58 °C for 60 s. After PCR, melting curves were acquired by stepwise increase of the temperature from 55 to 95 °C to ensure that a single product was amplified in the reaction.

From the same RNA samples used to assess transcriptional activity, quantitative real-time PCR was used to determine the relative amount of SNAT2 mRNA in each sample. RNA reaction mixtures were incubated at 48 °C for 30 min followed by 95 °C for 15 min and amplification of 35 cycles at 95 °C for 15 s, and then 60 °C for 60 s. To establish that a single product was amplified during the reaction, melting curves were generated by a stepwise increase of the temperature from 55 to 95 °C and measurements were taken at every degree change. Reactions were also run without RT to ensure that there was no DNA amplification. The primers used for PCR amplified the exon 10 region of SNAT2 mRNA and were as follows: human sense primer, 5'-CAGGTACAAGA-GCTGTTGGCTGTGT-3', and human antisense primer, 5'-GTG-TCTCTGTGGAAGCTGCTTTGA-3'; mouse sense primer, 5'-CT-CCTCCTCAAGACTGCCAACGA-3', and mouse antisense primer, 5'-TTCCAGCCAGACCATACGCCTTA-3'. The house-keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a negative control for starvation and as an indicator of the variation for the quantitative real-time PCR analysis. The primers used to measure relative mRNA levels for GAPDH were: sense primer, 5'-TTGGTATCGTGGGAAGGAC-TTC-3', and antisense primer, 5'-ACAGTCTTCTGGGTGGCAGT-3'. The results are expressed as arbitrary units of SNAT2 or GAPDH RNA relative to an RNA standard curve. The PCR reactions were performed in duplicate for each sample, and samples were collected from at least three independent experiments. The means $\pm$ S.E.M. were compared by Student's *t* test.

ChIP assays

ChIP analysis was performed according to our previously published method [17]. The reaction mixtures were incubated at 95 °C for 15 min, followed by amplification at 95 °C for 15 s and either 60 °C (SNAT2 enhancer primers) or 62 °C (SNAT2 promoter primers) for 60 s for 35 cycles. The SNAT2 promoter primers were: sense primer, 5'-GCCGCCTTAGAACGCCTTTC-3', and antisense primer, 5'-TCCGCCGTGTCAAGGGAA-3'. The SNAT2 enhancer primers were: sense primer, 5'-GGGAAGACGAGTTGGGAACATTTG-3', and antisense primer, 5'-CCCTCCTATGTCCGGAAAGAAAAC-3'.

Figure 1 Analysis of SNAT2 transcriptional activity and steady-state mRNA in HepG2 human hepatoma cells following amino acid deprivation

HepG2 cells were incubated for 0–24 h in MEM or in MEM lacking histidine (MEM – His). At the times indicated, RNA was isolated and analysed by quantitative RT–PCR. The transcriptional activity was determined by measurement of SNAT2 hnRNA using primers spanning the exon 4–intron 4 junction, whereas the steady-state mRNA levels for SNAT2 and GAPDH were assayed using primers within the protein coding sequence, as described in the Materials and methods section. The results are expressed in arbitrary units as the amount of SNAT2 or GAPDH RNA relative to an RNA standard curve. The results are represented as a summary of three independent experiments, each measured in duplicate, from which the results are depicted as the means $\pm$ S.E.M. Where not shown, the error bars are contained within the symbol.

RESULTS

Transcriptional activity and steady-state SNAT2 mRNA content after amino acid limitation

Previously published results documented that the elevation in SNAT2 mRNA was not due to stabilization, but rather, increased transcription [13]. Measurement of SNAT2 hnRNA was used to analyse the transcriptional activity from the SNAT2 gene. Within 2 h after histidine removal, transcription was increased and continued to rise until reaching a peak of three to four times the MEM control value at 8–12 h, and then it slowly declined over the next 12 h (Figure 1). When the SNAT2 steady-state mRNA content was monitored in the same samples (Figure 1), the level increased over the initial 12 h of amino acid limitation, but lagged slightly behind the increase in transcription in a consistent manner. For those cells maintained in complete MEM for 12 h or more, there was also a gradual increase in transcription and mRNA accumulation, a result that is consistently observed for this and other amino acid-regulated genes, and assumed to be the result of amino acid depletion of the culture medium. As a negative control, it was shown that amino acid limitation had little or no effect on GAPDH mRNA (Figure 1).

GCN2 and eIF2 α are required for activation of the SNAT2 gene

At least one component of the AAR pathway is the activation of GCN2 kinase, which, in turn, phosphorylates eIF2 α on Ser⁵¹ to trigger a reduction in global translation, but an increase in translation of ATF4 mRNA [10,28]. To establish that this pathway contributed to the induction of the SNAT2 gene, MEFs from GCN2-deficient mice or mice with an eIF2 α S51A mutation were incubated in complete MEM or MEM lacking histidine for 8 h. For the GCN2 wild-type fibroblasts, histidine deprivation resulted in an increase in SNAT2 mRNA of 1.6 ± 0.2 times the control. In contrast, there was no difference in SNAT2 mRNA expression in the GCN2^{−/−} fibroblasts; relative to the MEM control (set at 1.0), the MEM – His value was 1.0 ± 0.1 . Likewise, histidine limitation of MEF cells expressing an S51A mutated eIF2 α did not result in an increase in SNAT2 mRNA (MEM – His value was 0.8 ± 0.1 times the MEM control), whereas the corresponding wild-type fibroblasts exhibited an SNAT2 mRNA value that was 2.4 ± 0.3 times the MEM control. These results document that the AAR pathway is responsible for activation of the SNAT2 gene, and suggest that increased ATF4 production may be an important component.

ATF4 activates the transcription from SNAT2 genomic fragments

To demonstrate functional action of ATF4, transient expression of exogenous ATF4 was performed in HepG2 human hepatoma cells to monitor its effects on SNAT2-driven transcription using a firefly luciferase reporter gene. Two SNAT2-driven luciferase reporter constructs were employed, the SNAT2 – 512/+ 770 wild-type sequence, and the – 512/+ 770 sequence with the AARE mutated (Figure 2A). The control cells ('Vector') exhibited the expected increase in SNAT2 mRNA after incubation in histidine-free medium (Figure 2A). When cells were transfected with wild-type ATF4 and then maintained in complete MEM medium (open bars), there was a 54-fold increase in transcription. If these ATF4-transfected cells were incubated in the histidine-depleted medium (filled bars), the amino acid deprivation resulted in only a small additional increase. In contrast, when the SNAT2 construct with a mutated AARE was tested (Figure 2A, right panel), there was little or no increase in transcription following co-expression of wild-type ATF4 or after histidine deprivation (Figure 2A). Additionally, overexpression of a dominant-negative ATF4 isoform led to a complete blockade of the amino acid deprivation response (Figure 2A, left panel). These results show that elevated ATF4 levels result in activation of SNAT2-driven transcription and that this response requires an intact AARE.

Effect of other ATF transcription factors on SNAT2-driven transcription

ATF2 is required for induction of the CHOP gene following amino acid limitation [16,29] and multiple isoforms of ATF3 have been shown to influence the amino acid-dependent regulation of the ASNS gene [18]. To investigate the potential role of these two ATF factors in regulating the SNAT2 gene, they were transiently co-expressed in HepG2 cells with the SNAT2-driven (nt – 512/+ 770) luciferase reporter. Expression of ATF2 had no significant effect on the SNAT2-driven reporter gene transcription in the presence of complete MEM. However, there was a small, but statistically significant, stimulatory effect comparing the control cells ('Vector') and the ATF2-expressing cells, when both were incubated in histidine-depleted medium (Figure 2B). This result is consistent with the hypothesis by Averous et al. [16] that amino acid limitation causes phosphorylation of ATF2 and that this phosphorylation is a prerequisite for its action on the CHOP gene.

Figure 2 Expression of exogenous ATF4 regulates SNAT2-driven reporter gene transcription

HepG2 cells were transfected with a firefly luciferase reporter gene driven by either a wild-type SNAT2 genomic fragment (nt -512/+770) or the same construct with the AARE mutated (**A**). The mutations within the AARE sequence were the same as those shown in Table 1 for the EMSA oligonucleotides. The cells were co-transfected with expression plasmids for ATF4 wild-type (WT), ATF4 dominant-negative (DN) or empty pcDNA3.1 vector as control. (**B**) The Figure shows the results from parallel experiments in which the co-transfected expression plasmids contained sequences for ATF2, or the ATF3 isoforms ATF3-FL, ATF3ΔZip2c or ATF3ΔZip3. The cells were transferred, 16 h after transfection, to fresh MEM or medium lacking histidine for 10 h, the lysates were collected and the luciferase activities were measured. The control labelled 'Vector' (transfected with empty pcDNA3.1) MEM value was set to 1 and all other values were recalculated accordingly. Each data point represents the mean for six independent experiments. Error bars correspond to the means $\pm$ S.D. Statistically significant differences ($*P \leq 0.05$), indicated by an asterisk, are comparisons relative to the appropriate vector-only control. Therefore the MEM - His value for the cells transfected with empty vector is compared with the empty vector MEM value. For those cells that were transfected with a transcription factor, the MEM values (open bars) are compared with the vector-only MEM value, and the MEM - His values (filled bars) are compared with the vector-only MEM - His value.

When amino acid supply was sufficient (MEM condition), expression of ATF3-FL (full-length isoform of ATF3) led to a relatively small, but statistically significant, increase in the reporter gene expression, but the truncated ATF3 isoforms, ATF3ΔZip2c and ATF3ΔZip3, had no effect (Figure 2B). However, when the control and ATF3-expressing cells were deprived of histidine, expression of ATF3-FL completely blocked the induction of SNAT2-driven transcription, whereas ATF3ΔZip3 and ATF3ΔZip2c had no significant effect (Figure 2B). The strong repression by ATF3-FL was also observed when SNAT2 transcription was induced by expression of exogenous ATF4 rather than histidine limitation (results not shown).

Figure 3 The effect of exogenous C/EBP transcription factor expression on the transcription mediated by the SNAT2 CAAT/AARE

HepG2 cells were transfected with one of the following expression plasmids: C/EBPα, C/EBPβ-LAP or C/EBPβ-LIP (**A**) or C/EBPδ (**B**). The control cells were transfected with empty pcDNA3.1 plasmid. The co-transfected reporter constructs were: pGL3SV40-promoter alone, pGL3SV40-promoter with the wild-type CAAT/AARE sequence (nt +689 to +730) inserted 5' to the SV40 promoter (at the BglII site) or pGL3SV40-promoter with a mutant CAAT/AARE sequence (see Table 1 for mutation). The cells were transferred, 16 h after transfection, to fresh MEM medium for 10 h, the lysates were collected and the luciferase activities were measured. Each data point represents the mean for six independent experiments $\pm$ S.D. The asterisks ($*P \leq 0.05$) indicate statistically significant differences compared with the appropriate vector-only control values, as explained in the legend to Figure 1.

Regulation of SNAT2-driven transcription by C/EBP proteins

To examine the potential role of C/EBP proteins in the regulation of the SNAT2 gene, a transient transfection experiment was performed with either the wild-type SNAT2 CAAT/AARE (nt +689 to +730) or the same sequence with both the CAAT and the AARE sites mutated. These sequences were inserted 5' to the SV40 promoter in the pGL3-promoter firefly luciferase reporter plasmid and co-transfected with expression plasmids for C/EBPα, C/EBPβ-LAP (LAP isoform of C/EBPβ), C/EBPβ-LIP or C/EBPδ (Figure 3). Consistent with its action as an enhancer [13], even without exogenous C/EBP expression ('Vector'), the presence of the wild-type CAAT/AARE sequence conferred amino acid responsiveness to the SV40 promoter (Figure 3A, top panel), whereas regulated transcription from its mutated counterpart was blocked (Figure 3A, middle panel). The co-expression of

exogenous C/EBP α or C/EBP β -LAP led to enhanced transcription in cells maintained in amino acid complete medium (open bars) and no further increase was observed in cells deprived of histidine (filled bars). Expression of the naturally occurring dominant-negative isoform, C/EBP β -LIP, completely blocked the induction after amino acid limitation (Figure 3A, top panel). When the reporter gene was driven by the SNAT2 sequence with both the CAAT and AARE mutated, the effect of amino acid limitation was blocked as expected (Figure 3A, middle panel, 'Vector'). The stimulation of transcription by either C/EBP α or C/EBP β -LAP was detectable, but the magnitude was greatly reduced (Figure 3A, middle panel), and furthermore, the residual minor increases remaining were also observed when a control construct containing the SV40 promoter alone was tested (Figure 3A, bottom panel). C/EBP δ was identified as a positive clone during a yeast one-hybrid screen for SNAT2 AARE binding proteins (results not shown). Expression of exogenous C/EBP δ caused a significant reduction in the CAAT AARE-mediated induction of transcription in response to histidine limitation (Figure 3B, compare filled bars for Vector versus C/EBP δ). In contrast, similar experiments performed with C/EBP γ expression showed no significant effect on SNAT2-driven transcription (results not shown). These results suggest that for those tissues in which it is expressed, C/EBP δ may act as a repressor of nutrient control of SNAT2 expression.

The CHOP and ASNS AARE sequences compete for SNAT2 AARE binding proteins

The 9 bp core sequence of the SNAT2 AARE is identical with the AARE in the CHOP gene [13], and only 2 nt different from that in the ASNS gene [12]. However, the flanking sequences for all three of these sites are different, and from ASNS and CHOP AARE swapping experiments, there is evidence that the flanking sequence influences the activity [14]. To investigate whether or not these three elements share common binding proteins, the CHOP and ASNS AARE sequences were used as unlabelled competitors for the SNAT2 AARE probe in an EMSA study (Figure 4). The oligonucleotide sequences used in all EMSA experiments are given in Table 1. At least three SNAT2-associated complexes were detected, and their abundance was increased when nuclear extracts from histidine-deprived cells were tested (Figure 4). The unlabelled SNAT2 AARE sequence ('SNAT2, 200X') was used as a specific competitor to demonstrate that all three complexes represent specific binding. In some gels, the third complex was resolved into a doublet, as illustrated in later Figures in the present study. When the CHOP and ASNS AARE oligonucleotides were present as competitors, they caused a reduction of all three complexes assembled on the SNAT2 AARE. Although the SNAT2 and CHOP 9 bp core sequences are the two that are identical, a 200-fold excess of the ASNS AARE completely abolished the SNAT2-associated complexes, whereas the same amount of the CHOP AARE was not as effective (Figure 4). It is clear from these results that the three AARE sites share common binding proteins, but they may differ in their relative affinity.

Proteins that bind to the SNAT2 AARE also have affinity for the SNAT2 CAAT box

The ASNS, CHOP and SNAT2 AARE sequences are referred to as C/EBP-ATF composite sites, because each is made up of a half-site for C/EBP consensus binding and a half-site for the ATF family [30]. In the case of the ASNS site, binding of C/EBP β has been shown to occur *in vitro* [20] and *in vivo* [17]. In contrast with CHOP and ASNS, the SNAT2 gene also has a CAAT-like palindromic sequence 11 bp upstream from the AARE (Figure 5A), which is not absolutely necessary for induction

Figure 4 Proteins bound to the SNAT2 AARE are in common with those bound to the CHOP and ASNS AARE sites

Prior to nuclear extract isolation, HepG2 cells were incubated for 4 h in either complete MEM or MEM lacking histidine (– His), both supplemented with 10% dialysed FBS. EMSA analysis to monitor binding to the SNAT2 AARE was performed as described in the Materials and methods section. Where indicated, an unlabelled oligonucleotide, corresponding to the sequences surrounding the human CHOP or ASNS AARE sequences, was added at 1–200 times the amount of the radiolabelled SNAT2 AARE oligonucleotide probe. The probe and competitor sequences are listed in Table 1. The arrows marked I, II and III denote specific complexes that were increased in amount when extracts from histidine-deprived cells were tested. The autoradiographic film shown is representative of several separate experiments using independently prepared nuclear extracts.

after amino acid limitation, but which does enhance the response [13]. The potential for C/EBP proteins to bind to both of these elements prompted tests by EMSA to determine if an excess of the SNAT2 CAAT box sequence would compete away the specific complexes assembled at the AARE (Figure 5B). As expected, the presence of an excess of the unlabelled wild-type SNAT2 AARE completely abolished the formation of the specific complexes (Figure 5B), whereas a mutated AARE sequence had little or no effect. An excess of the wild-type SNAT2 CAAT box produced a detectable amount of competition for the AARE-binding proteins (Figure 5B), whereas the corresponding oligonucleotide with the CAAT box mutated produced minimal competition. These results suggest that the SNAT2 CAAT box and AARE sites may share proteins in common.

Transcription factors present in the SNAT2 AARE protein–DNA complexes

Studies using both *in vitro* EMSA [15,20] and *in vivo* ChIP analysis [17] have shown that ATF4 and C/EBP β bind to the ASNS AARE site. EMSA supershift studies were performed to determine whether or not these two factors are present in the SNAT2 AARE-associated complexes induced by amino acid limitation (Figure 6). The results show that the inclusion of ATF4 antibody caused the appearance of a supershifted band that originated

Figure 5 Complexes formed at the SNAT2 AARE and the SNAT2 CAAT box contain proteins in common

(A) The sequence of the human SNAT2 intron 1 sequence, nt +692 to +730, containing the CAAT box and the AARE is shown. (B) Nuclear extracts were prepared from HepG2 cells incubated for 4 h in either complete MEM or MEM lacking histidine (MEM – His), both supplemented with 10% dialysed FBS. EMSA analysis to monitor binding to the SNAT2 AARE was performed using a 21 nt AARE probe. Where indicated, a 100 times amount of either wild-type or mutated oligonucleotide containing either the SNAT2 AARE or the SNAT2 CAAT box was added. The probe and competitor sequences are listed in Table 1. The arrows marked I, II and III denote specific complexes that were increased in amount when extracts from histidine-deprived cells were tested. The autoradiographic film shown is representative of several separate experiments using independently prepared nuclear extracts.

primarily from complex III and that was readily observed when extracts from amino acid-deprived HepG2 cells were used (Figure 6). In contrast, the presence of an antibody specific for C/EBP β caused a shift by all three primary complexes, although the shift for complex III was not complete (Figure 6).

ChIP analysis of the ASNS AARE [17] and EMSA using the CHOP AARE [16] has suggested that other transcription factors with leucine zippers may be present and that their binding may be influenced by amino acid limitation. Antibodies specific for ATF2, ATF3 and c-Fos produced little or no supershift of complexes assembled with either MEM or MEM – His extracts (Figure 7). For the extracts from cells lacking histidine, the c-Jun antibody reduced the overall intensity of all three specific complexes, but no discrete supershifted bands were observed (Figure 7, lower panel). There was a slight increase in the amount of a C/EBP δ -containing complex when extracts from histidine-deprived cells were compared with control (MEM) cells. C/EBP α binding was detected as a complete retardation of complex I and a partial shift

Figure 6 Complexes formed at the SNAT2 AARE contain ATF4 and C/EBP β

Nuclear extracts derived from HepG2 cells exposed to complete MEM or MEM lacking histidine (MEM – His) for 4 h were used to monitor transcription factor binding. Specific binding was shown by the presence of a 100-fold excess of the unlabelled probe ('Specific') and non-specific binding was monitored by the addition of a 100-fold excess of an unrelated ('Non-spec') oligonucleotide (sequences given in Table 1). A fourth complex, that ran faster than complex III, was detected in this particular experiment, but it was not present in most extracts tested. The addition of antibody specific for either ATF4 or C/EBP β was as described in the Materials and methods section, and the supershifted complexes are indicated (●).

of complex II in both control (MEM) and amino acid-deprived cells (Figure 7).

ATF4 binds to SNAT2 AARE as a heterodimer

To further investigate the potential role of ATF4 protein in the regulation of the SNAT2 gene, a series of preliminary experiments with purified ATF4 protein was performed to optimize the conditions. When a nuclear extract titration experiment was performed in the presence of a constant amount of ATF4 protein, it was observed that three main complexes were formed and that saturation was reached with an extract protein content of 2.5 μ g or higher (results not shown). So, 3 μ g of nuclear extract from cells incubated in either MEM or MEM lacking histidine was used to titrate the optimal amount of ATF4 protein, which was shown to be linear from 50 to 125 ng. The total abundance of the complexes formed was always greater in the histidine-deprived cells (results not shown). This latter result suggests that the nuclear abundance of potential ATF4 partners is increased following amino acid limitation.

To investigate the identity of the transcription factors that are bound to the SNAT2 AARE along with ATF4, a supershift analysis with antibodies against related transcription factors was performed in the presence of a constant amount of purified ATF4 protein (50 ng) and nuclear extract (3 μ g) from cells deprived of histidine for only 40 min to keep the increase in endogenous ATF4 relatively small and to investigate the initial complexes formed [17]. The results, illustrated in Figure 8 (lanes 1–9), document that three primary complexes were formed and that ATF4 is present in all three. C/EBP α was present in complex I, whereas antibodies against C/EBP β and c-Jun both caused the supershift of a relatively weak band (Figure 8), the origin of which could not be definitively established, but the abundance

Figure 7 The protein complexes formed at the SNAT2 AARE contain factors other than ATF4 and C/EBP β

Nuclear extracts derived from HepG2 cells exposed to complete MEM or MEM lacking histidine (MEM – His) for 4 h were used to monitor transcription factor binding. Specific binding was shown by the presence of a 100-fold excess of the unlabelled probe ('Specific') and non-specific binding was monitored by the addition of a 100-fold excess of an unrelated ('Non-spec') oligonucleotide (sequences in Table 1). The addition of antibody specific for the transcription factors indicated was as described in the Materials and methods section, and the supershifted complexes are indicated (●).

of complex I appeared to be reduced. Antibodies against C/EBP δ , ATF2 and ATF3 did not produce detectable supershifted bands. These experiments suggest that ATF4 protein binds to the SNAT2 AARE sequence as a heterodimer in concert with factors such as C/EBP α , C/EBP β and c-Jun, but also demonstrate that ATF4 associates with unidentified proteins to generate complexes II and III.

To further characterize these ATF4-containing complexes, an excess (100 times) of unlabelled competitor oligonucleotide was included in the binding reaction (Figure 8, lanes 10–13). An excess of unlabelled SNAT2 AARE probe itself or an oligonucleotide containing the CHOP AARE sequence was equally efficient in competing away the specific complexes. Conversely, a palindromic ATF consensus sequence caused little or no change in the abundance of the ATF4–AARE complex, suggesting that under these circumstances, a sequence requiring ATF4 homodimerization was not an effective competitor. In contrast, the presence of a palindromic C/EBP consensus site caused a significant reduction in all three ATF4-containing complexes (Figure 8).

Transcription factor binding to the SNAT2 gene *in vivo*

To investigate *in vivo* binding of factors to the SNAT2 gene, ChIP assays were performed to monitor protein interactions at both

Figure 8 Identification of potential ATF4 partners analysed by binding of purified ATF4 protein to the SNAT2 AARE

A nuclear extract was prepared from cells deprived of histidine for 40 min, a time that allows for the initial induction of the SNAT2 transcription to begin [17]. A 50 ng aliquot of purified ATF4 protein was added to each incubation. Some incubations contained antibodies specific for the indicated transcription factors (lanes 1–9). A 100-fold excess of oligonucleotides (Oligo competitor) containing ATF or C/EBP consensus sequences, or the SNAT2 or CHOP AARE sequences were added to test for competition (lanes 10–13). The three specific complexes are designated as I–III. The ATF2 antibodies labelled 1 and 2 are against the basic region and the C-terminus of the protein respectively.

the SNAT2 promoter and the intronic AARE enhancer regions (Figure 9). As expected, there was increased binding of RNA Pol II at both the SNAT2 enhancer regions (Figure 9A) and promoter (Figure 9B) as early as 3 h following histidine removal, and the elevated binding was maintained for up to 12 h. In contrast with Pol II, there was little or no binding of ATF4, ATF3, C/EBP α or C/EBP β to the SNAT2 promoter region before or after amino acid deprivation. Given that these results were negative, only the results for ATF4 are shown as an example in Figure 9(B). However, after histidine removal from the medium, there was a large and relatively rapid increase in ATF4 bound to the SNAT2 intronic AARE region (Figure 9A). The kinetics of ATF4 binding during the initial 4 h appeared to precede those for Pol II binding to the promoter. As detected for the transcriptional activity and mRNA content (Figure 1), after 12 h there was also a gradual increase in ATF4 and C/EBP β binding in those cells maintained in complete MEM, probably the result of amino acid depletion of the culture medium. Approximately 12 h after histidine limitation, at a time when the transcription activity from the gene was declining (Figure 1), the binding of ATF3 began to increase and reached a peak at approx. 16 h (Figure 9A). This result is consistent with the ATF3 functional analysis (Figure 2) and the proposal that ATF3-FL is a repressor of AARE-mediated transcription [17,18]. In contrast with both ATF4 and ATF3, there was a significant amount of constitutive C/EBP β binding in the control cells (time = 0 h) and cells maintained in complete MEM medium (Figure 9A). After transfer of the HepG2 cells to MEM lacking histidine, the C/EBP β binding to the SNAT2 AARE region increased during the initial 12 h of amino acid limitation and then slowly declined

Figure 9 *In vivo* transcription factor binding to the SNAT2 promoter and the AARE enhancer

HepG2 cells were incubated in either the complete MEM or MEM lacking histidine (MEM – His) for 0–24 h. ChIP analysis was performed using the antibodies indicated against non-specific rabbit IgG (results not shown), ATF4, ATF3, C/EBP α , C/EBP β , C/EBP δ or RNA Pol II. Quantitative PCR was used to analyse the relative binding to either the SNAT2 AARE intronic enhancer (**A**) or to the proximal promoter (**B**). Data were plotted as the ratio to the value obtained with a 1:20 dilution of input DNA. Each point represents the mean value for three independent experiments, and the error bars represent the S.E.M. Within each experiment, a non-specific rabbit IgG was used to establish the background for the immunoprecipitation. These values were always less than 0.01 (calculated as the ratio to input DNA) and no differences between the MEM and MEM – His conditions were observed.

(Figure 9A). Binding of C/EBP α and C/EBP δ was detectable, but the amount was highly variable in both the MEM and MEM – His conditions. The reasons for this variation, although observed consistently between multiple experiments, are unknown, yet the presence of C/EBP α is consistent with the functional analysis showing activation by exogenous expression (Figure 3) and the EMSA results showing C/EBP α binding activity in nuclear extracts (Figure 7). Likewise, the presence of C/EBP δ is in line with the binding observed by EMSA in nuclear extracts from MEM – His cultured cells (Figure 7). Further analysis of the role of C/EBP α and C/EBP δ will be required to understand the changes observed.

DISCUSSION

The results described in the present study illustrate the involvement of specific transcription factors in the regulation of human SNAT2 gene expression following amino acid deprivation, and document several novel observations. (i) The SNAT2 AARE enhancer shares common binding proteins with the ASNS and CHOP AARE and the nearby SNAT2 CAAT box. (ii) The protein complexes formed at the SNAT2 AARE are increased in abundance following amino acid deprivation, and specific ATF and C/EBP transcription factors are detected in these complexes by EMSA supershift analysis. (iii) ATF4 was detected in the same

complexes with C/EBP α and C/EBP β , with which it probably partners in the regulation of the SNAT2 transcription mediated by the AARE. (iv) Transcription from the SNAT2 gene was strongly enhanced by the transient expression of wild-type ATF4, and it required an intact AARE. In contrast, expression of an ATF4 dominant-negative isoform, ATF3-FL, C/EBP β -LIP and C/EBP δ antagonized the activation of the SNAT2 gene in response to amino acid deprivation. (v) Using nuclear extracts from amino acid-deprived cells, ATF4, C/EBP α , C/EBP β and C/EBP δ physically associated with the SNAT2 AARE sequence and showed amino acid-dependent changes in binding, and ChIP analysis largely confirmed the observations by EMSA, although some differences were noted. (vi) Transient expression of these transcription factors provided functional evidence for their role in the regulation of the SNAT2 gene. (vii) ChIP analysis also documented an amino acid-regulated association of RNA Pol II with both the promoter and enhancer regions of the SNAT2 gene.

In contrast with the SNAT2 AARE, which is absolutely required to detect any induction by amino acid limitation, when the nearby CAAT box is mutated the activated transcription is reduced by approx. 40% [13]. The EMSA results included in the present study demonstrate that there are proteins in common in the complexes that bind to the AARE and the CAAT box. It is interesting to note that the AAREs among the identified amino acid responsive genes comprise different components. As mentioned, the ASNS gene absolutely requires both of two distinct elements that are separated by one turn of DNA [12,22]. Bannai and co-workers [31] showed that the anionic amino acid transporter xCT gene is amino acid-regulated and contains two identical AARE sequences (5'-TGATGCAA-3'), also one turn of DNA apart. Although they are present in opposite orientations, both are required for full induction of transcription [31]. Ohoka et al. [32] have identified an AARE-like sequence in the TRB3 (tribbles-related protein 3) gene that is ATF4-responsive. Interestingly, 7–9 bp upstream of the TRB3 AARE is a CAAT-like sequence that is required for activation of the gene by tunicamycin-induced endoplasmic reticulum stress, but not by overexpression of ATF4. In contrast, the CHOP AARE appears to function independently of any additional elements [14]. Further investigation of these and other amino acid-responsive genes will be required to understand fully the mechanistic and functional differences between these single-element and bipartite AARE sites.

The present observations provide *in vivo* evidence for transcription factor binding to the intronic AARE and illustrate the key role that ATF4 plays in activating the SNAT2 gene. The ATF4 association with the SNAT2 intronic enhancer region prior to RNA Pol II binding at the promoter suggests that ATF4 may be a component of the trigger mechanism for the SNAT2 activation, promoting further recruitment of activator proteins to the promoter and enhancer regions. Similar to the activation of the ASNS gene [17], the SNAT2 stimulation in response to amino acid-limiting conditions is also subject to a self-limiting programme, whereby the activation by ATF4 appears to be transitory because of subsequent binding of repressor proteins such as ATF3. Amino acid limitation causes an induction of transcription [18] and mRNA stabilization [33] for the transcription factor ATF3. As a component of the increased transcription, there is alternative splicing of the resulting transcripts such that there is an increase in three mRNA species that code for a full-length protein (ATF3-FL) and two truncated isoforms, ATF3 Δ Zip2c and ATF3 Δ Zip3 [18]. Consistent with its proposed function as a transcriptional repressor [34], when overexpressed, ATF3-FL inhibited the induction of ASNS transcription by ATF4 expression or amino acid deprivation [18]. Conversely, expression of the ATF3 Δ Zip2c isoform had no effect, and expression of ATF3 Δ Zip3 actually resulted in a further

increase in ATF4-induced and starvation-induced ASNS-driven transcription. The lack of an effect of the ATF3 Δ Zip3 isoform on SNAT2 AARE-driven transcription is in clear contrast with its activation via the ASNS AARE [18]. If the latter effect is the result of sequestering co-repressors, as hypothesized by Hai and co-workers [35], the present results would suggest that the co-repressors associated with the ASNS and SNAT2 AARE sites are different.

Collectively, the results presented in this paper, both *in vitro* and *in vivo*, document that multiple transcription factors bind to the SNAT2 AARE and contribute to the amino acid-dependent regulation of this physiologically important amino acid transport activity. The similarity of the factors that control SNAT2 transcription and those that have been shown previously to activate the ASNS gene, illustrate that both amino acid uptake and amino acid synthesis may be regulated in concert to contribute to the cellular response to this nutritional challenge. The identification of these transcription factors and delineation of their binding kinetics to AARE sequences provide the basis for future studies designed to understand how these factors themselves are regulated by the AAR pathway.

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