Transcriptional Control of the Human Sodium-coupled Neutral Amino Acid Transporter System A Gene by Amino Acid Availability Is Mediated by an Intronic Element*

Stela S. Pali, Hong Chen, and Michael S. Kilberg†

From the Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, Florida 32610

System A amino acid transporter (SNAT2) gene expression is up-regulated at the transcriptional level in response to amino acid deprivation. Functional analysis of genomic fragments 5' upstream of the transcription start site, for both human and mouse SNAT2 genes showed that these regions exhibit promoter activity, but were amino acid unresponsive. However, when the human and mouse constructs were extended to include intron 1, it was observed that the rate of transcription was increased following amino acid deprivation. Deletion analysis of the human gene identified an intron 1 sequence spanning 54 nucleotides that was sufficient for conferring amino acid-dependent regulation to a minimal SNAT2 promoter. Alignment of the corresponding region from the human, mouse, and rat genomes revealed three highly conserved sequences. From site-directed mutagenesis, it was concluded that one of these sites functions as a amino acid response element (AARE) to regulate transcription. The core sequence of this site is identical to the AARE in the human CHOP gene. The SNAT2 AARE, along with a nearby conserved CAAT box, has enhancer activity in that it functions in an orientation and position independent manner, and it confers regulated transcription to a heterologous promoter.

System A amino acid transport activity is sodium-dependent, pH-sensitive, inhibited by high concentrations of intracellular amino acids, and displays a preference for neutral amino acids with unbranched side chains. There are a number of recent reviews that have summarized the current understanding of amino acid transport mechanisms and transporter regulation (1–4). System A can be the rate-determining step for hepatic alanine utilization and may also contribute to whole body nitrogen homeostasis as a mediator of glutamine transport (1, 2). The activity is linked to the cell cycle and cell growth rate as illustrated by the observation that the transformed cells exhibit subsequently elevated system A uptake relative to parental cells or tissues (5). System A transport activity is up-regulated in mammalian cells in response to amino acid deprivation (6, 7), but until recently, little progress was made toward understanding the molecular mechanisms underlying this increase because of the lack of molecular tools. Indirect evidence suggested that the gene encoding System A is regulated at the level of transcription (8).

During the past several years cDNA sequences for three System A-encoding isoforms have been cloned (9–13) and the nomenclature SNAT1, 2, and 4 (sodium-coupled neutral amino acid transporter) has been proposed (14). Recently, it was shown that human SNAT2 is the primary isoform induced by amino acid deprivation and there is a direct relationship between SNAT2 mRNA expression and System A transport activity (15, 16). Extending these studies, we recently documented amino acid-regulated transcription from a human SNAT2 genomic fragment (17). A decline in cellular amino acid content results in enhanced translation of specific mRNA species (18) and elevated transcription of selected genes. A number of examples of this increased transcription have been reported for genes encoding enzymes, transporters, and ribosomal proteins (19, 20). One example is the human asparagine synthetase gene for which transcription increases in response to either amino acid deprivation or endoplasmic reticulum stress (21–23). It has been demonstrated that nutrient sensing response element-1 and -2 (NSRE-1 and NSRE-2),1 located in the asparagine synthetase proximal promoter, mediate both of these responses (24). The human C/EBP homology protein (CHOP) promoter contains a sequence that differs from the asparagine synthetase NSRE-1 by only two nucleotides (5'-ATGATGCAAT-3') and functions as an amino acid response element (AARE) (25, 26). The CHOP AARE has been termed a C/EBP-ATF composite site, because it binds members from both of these subfamilies of the bZIP superfamily of transcription factors (27, 28).

The molecular mechanism for increased SNAT2 mRNA production following amino acid deprivation has been contrasted with that for asparagine synthetase (17), and fundamental differences were observed between the mechanisms for nutrient regulation. For example, when HepG2 hepatoma cells were incubated in amino acid-deprived media, there was a lag period of ~4 h before an increase in asparagine synthetase mRNA content was detected, whereas the elevation of SNAT2 mRNA was detectable as early as 2 h following amino acid removal. Additionally, de novo protein synthesis was absolutely required for the asparagine synthetase activation, whereas the increase in SNAT2 mRNA content was largely independent of protein synthesis. Based on these observations, it was concluded that it is unlikely that the SNAT2 gene will contain the same genomic

1 The abbreviations used are: NSRE, nutrient sensing response element; SNAT2, sodium-coupled neutral amino acid transporter-2; CHOP, C/EBP homology protein; nt, nucleotide(s); AARE, amino acid response element; MEM, minimal essential medium; C/EBP, CCAAT/enhancer-binding protein.
nutrient-responsive cis-elements as the asparagine synthetase gene

The purpose of this paper is to report the identification of the SNAT2 AARE, describe its initial characterization, and document its unique localization within the first intron for both the human and the mouse genes. In close proximity to the AARE, there are two highly conserved sequences that contribute to regulated SNAT2 expression. One is a purine-rich (PuR) sequence. When appropriate, the oligonucleotides were designed with Kpn restriction enzyme overhangs (not listed) and this feature was used to facilitate subsequent cloning into the respective sites of the vector, as described under “Materials and Methods.” All sequences correspond to the human gene, unless indicated as “mouse.” Mutations are shown as bold sequence.

### Table I

| Clone | Primers |
|-------|---------|
| +222/+1418, mouse | Forward: 5'-CCGGCAACAGGACAAAGAAA-3'  
Reverse: 5'-GAAAGCCCAAGGATTCAC-3' |
| -227/+1465, mouse | Forward: 5'-GGATGGGCTCGGTAATT-3'  
Reverse: 5'-GAAGCTTCGAGCAAGGAGG-3' |
| -1111/+59 | Forward: 5'-GGCCCTGGCAATTCTTCCTC-3'  
Reverse: 5'-GTTGGGCCGTCGCTAGCAG-3' |
| -512/+59 | Forward: 5'-TCAGGACACCGCGCTCGCTTG-3'  
Reverse: 5'-GTCGGGCGTGCAGTACGAGG-3' |
| -222/-1 | Forward: 5'-GGGCTTTGCGGAGAGTCTGGGG-3'  
Reverse: 5'-GGAGCAGGCCTGGCAGGCG-3' |
| -218/+770 | Forward: 5'-CTTTGGGCAGGGAGTCTGGGGCGC-3'  
Reverse: 5'-CCCGCGAGTGCTACGCCCGC-3' |
| -512/+698 | Forward: 5'-TCAGGACACCGCGCTCGCTTG-3'  
Reverse: 5'-GTCGAATGGTCTCCAACTC-3' |
| AARE | Forward: 5'-CTTAGGGACATGGAATAGTCTGGAGG-3'  
Reverse: 5'-CATGGGAAAGAAAACTGCAATATCGATCGTGG-3' |
| CAAT/AARE | Forward: 5'-CATGGACATTTGCAACTGAGATCAGATGCAGTCTGAGTCCATGAGG-3'  
Reverse: 5'-GAAGAACTAGTGCAATATCGATCGTGGTGCACATGAGG-3' |
| +689/+754 | Forward: 5'-ACATTGGAATCGGATCGATAGTTTCTCTCAGGAGGGCTGGGCGG-3'  
Reverse: 5'-CCGCGCGACGCCCTCTATGCGGGAGAGTCTTGGGGCGC-3' |
| CAAT box mutant | Forward primer 5'-CGAGTGGAGCAACATTGCTCGGAGGAGG-3'  
Reverse primer: 5'-CCATGGCGGTACGATGCTGAC-3' |
| AARE mutant | Forward primer 5'-CGAGATCGATCGGAGGAGGAGG-3'  
Reverse primer: 5'-TGGGCGACGCGAGCTGCTGAC-3' |
| PuR box mutant | Forward primer 5'-CCCGAGATCGGAGGAGGAGGAGG-3'  
Reverse primer: 5'-CCCGCGCCAGAATCCTATGCGGTCC-3' |

### MATERIALS AND METHODS

**Cell Culture**—Human HepG2 hepatoma cells were cultured in 75-cm² tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO₂, 95% air. The medium used was Modified Eagle Medium (MEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 25 μg/ml streptomycin sulfate, 10 μg/ml gentamycin, 0.025 μg/ml N-buty1-p-hydroxybenzoate, 0.2% (v/v) bovine serum albumin, and 5% (v/v) fetal bovine serum. For amino acid deprivation experiments, the complete MEM was removed and replaced with either fresh complete MEM or MEM lacking histidine, each supplemented with 5% dialyzed fetal bovine serum.

**Northern Analysis**—Cells were grown to 60–70% confluence in 60-mm tissue culture dishes, subjected to the amino acid deprivation treatment as described in the figure legends, and then RNA was collected using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was subjected to Northern analysis as described by Barbosa-Tessmann et al. (22). The cDNA probes for the human SNAT2 and the ribosomal protein L7a were described previously (17). Radiolabeled cDNA probes were prepared with [32P]dATP (Amersham Biosciences) using a Strip-EZ DNA probe synthesis kit (Ambion, Austin, TX). Autoradiographs were made using Kodak Bio-Max film and Bio-Max MR intensifier screens (Kodak, Rochester, NY). The relative intensity of each experimental band was determined by densitometry and then normalized to the signal intensity of the L7a control mRNA for which there was little or no change in expression under the experimental conditions.

**Cloning of Genomic Fragments and Plasmid Construction**—Human and mouse SNAT2 genomic fragments, corresponding to the region 5’ upstream of the transcription start site, were amplified by PCR from a bacterial artificial chromosome 12 clone (number RP11-474P2 from the BACPAC Resource Center, Oakland, CA). The obtained PCR products were cloned in front of the promoter-less firefly luciferase reporter vector using either the KpnI or BglII sites of the pGL3 plasmid (Promega, Madison, WI). A mouse genomic library (number RPCI 23 from the BACPAC Resource Center) was screened using a DNA probe generated by PCR from mouse genomic DNA using primers based on the published human SNAT2 sequence with the forward primer starting at nt +1418 (in exon 2) and the reverse primer starting at nt +2224 (in exon 4) of the human gene (see Table I). Screening of the mouse genomic library yielded four positive clones, one of which, was used for further analysis. From the original positive, a 1.7-kb BamHI fragment and a 5-kb XbaI fragment, which hybridized to the mouse Snat2 probe, were obtained and then subcloned. Sequencing results showed that the BamHI fragment contained mouse Snat2 sequence from nt −227/+1465, based on a reported transcription start site (GenBank accession number AK017145). Likewise, it was shown that the XbaI fragment contained
atoma cells were seeded in 24-well plates at the density of 1.5 × 10^5 cells/well, supplied with complete MEM and grown for 24 h. Transfection was performed with 1 μg of plasmid DNA per 10^5 cells and the Superfect reagent (Qiagen, Valencia, CA) was present at a ratio of 1:6 g of DNA:g of reagent, according to the manufacturer's protocol. When appropriate, a human asparagine synthetase construct incorporating the minimal core promoter sequence, nt −1525/+3700 of the mouse Snat2 gene. Subsequently, smaller fragments were generated by restriction enzyme digestion, and a putative putative minimal core promoter sequence, nt −227/+1 was generated by PCR using primers (see Table I) that were based on sequencing the BamHI fragment. For the experiments designed to test the transcriptional regulatory properties of these genomic fragments, PCR products were cloned into a firefly luciferase reporter vector (pGL3-control vector) or a putative upstream promoter fragment of the human SNAT2 gene (nt −512/+59) to function as a basal, homologous promoter. The fidelity of the clones and their orientation were tested by restriction enzyme digestion and gel electrophoresis, and confirmed by sequencing.

**Transient Transfection and Luciferase Assays—**Human HepG2 hepatoma cells were seeded in 24-well plates at the density of 1.5 × 10^5 cells/well, supplied with complete MEM and grown for 24 h. Transfection was performed with 1 μg of plasmid DNA per 10^5 cells and the Superfect reagent (Qiagen, Valencia, CA) was present at a ratio of 1:6 g of DNA:g of reagent, according to the manufacturer's protocol. When appropriate, a human asparagine synthetase construct incorporating the minimal core promoter sequence, nt −115/+115 in front of the firefly luciferase was used as a positive control for the amino acid deprivation response (29). Each well also received 5 ng of pRL-SV40 plasmid (Renilla luciferase) to serve as a control for transfection efficiency. After 3 h, cells were rinsed once with phosphate-buffered saline and given fresh MEM. At 16 h post-transfection, the medium was removed, the cells were rinsed once with phosphate-buffered saline and incubated for 10 h in 1 ml/well of either complete MEM or MEM lacking histidine, both supplemented with 5% dialyzed fetal bovine serum. After the completion of treatment, the cells were rinsed with phosphate-buffered saline, lysed with 100 μl of 1× Passive lysis buffer (Promega), and then subjected to one freeze-thaw cycle to assure complete disruption of the membranes. Firefly and Renilla luciferase activities were measured in a Sirius luminometer (Berthold Detection Systems, GmbH, Germany) using the Dual Luciferase Reporter Assay System (Promega).

**Site-directed Mutagenesis—**To generate the mutant sequences, a plasmid containing the human SNAT2 sequence from nt −512/+770 was used as a wild-type template. The mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The identity and fidelity of the constructs was verified by sequencing. The mutagenic primers used are presented in Table I (the mutated nucleotides are in bold and underlined). To generate mutants for multiple sites, plasmids containing mutated sequences were used as templates for additional rounds of site-directed mutagenesis.

**Nuclear Extract Preparation and Electrophoresis Mobility Shift Assay—**HepG2 cells were seeded on 150-mm dishes (15 × 10^6 cells per dish). After 16 h of culture, the cells were washed twice with phosphate-buffered saline and incubated for 4 h in either complete MEM or MEM lacking histidine, both supplemented with 5% dialyzed fetal bovine serum. The nuclear extraction was performed as previously described (30). Protein concentration was determined using a modified Lowry assay (31). Single-stranded oligonucleotides were annealed by adding 4.8 nmol of each oligonucleotide, with 20 μl of 10× annealing buffer (100 mM Tris-HCl, pH 7.5, 1 M sodium chloride, 10 mM EDTA) in a total volume of 200 μl. The oligonucleotide solution was heated to 95°C for 5 min and then allowed to gradually cool to 4°C over 2 h. The oligonucleotides used as either electrophoresis mobility shift assay probes or competitors are listed in Table II. The double-stranded oligonucleotides were radiolabeled by extension of overlapping ends with Klenow fragment in the presence of [α-32P]dATP. For each binding reaction, 10 μg of nuclear extract protein was incubated with 40 μg Tris base, 200 mM NaCl, 2 mM dithiothreitol, 10% (v/v) glycerol, 0.05% (v/v) Nonidet P-40, 3 μg of poly(dI-dC) (Amersham Biosciences), 0.04 pmol of unrelated DNA (see Table II), and 0.05 mM EDTA for 20 min on ice. The radiolabeled probe was added at a concentration of 0.02 pmol/reaction (−20,000 cpm), and unlabeled competitor oligonucleotides were added at the indicated concentrations. The reaction mixture, 30 μl final volume, was incubated at room temperature for 20 min. The reactions were subjected to electrophoresis as described previously (30).

**Gene Structure and Sequence Alignments—**Alignments of the mammalian SNAT2 gene structures were generated by using the program “Spidey-mRNA to genomic alignment.” The GenBank accession numbers for SNAT2 sequences were: human, NT_029419 and AF288897; mouse, AC109201 and XM_128198.2; and rat, NW_044046.1 and XM_217051. The genomic sequences for the mouse, rat, and human

---

### Table I:

| Species | Pre-mRNA length | mRNA length | Translation start codon | Translation stop codon |
|---------|-----------------|-------------|------------------------|-----------------------|
| Human   | 14596 nt        | 4795 nt     | +109                   | +1164                 |
| Mouse   | 12335 nt        | 4656 nt     | +998                   | +9579                 |
| Rat     | 12164 nt        | 4632 nt     | +917                   | +8337                 |

---

Fig. 1. The SNAT2 gene structure in three mammalian species. In the **top panel**, the 16 exons are represented by the **vertical lines** and boxes, with the space between them illustrating the 15 introns. The exon/intron lengths are drawn to scale and reflect the relation within each species. The positions of the translation start (AUG) and stop (UAA) codons are indicated by the asterisks in exons 2 and 16, respectively. To obtain the data in the **bottom table**, the mammalian SNAT2 gene structures were generated by using the program Spidey-mRNA to genomic alignment as described under “Materials and Methods.” The translation codon positions are relative to the putative transcription start site (+1) for each gene.

Fig. 2. SNAT2 mRNA content in three human cell lines. Cells were incubated for 12 h in either complete MEM (MEM) or amino acid-free Krebs-Ringer bicarbonate buffer (−AA). Northern blot analysis with total mRNA extracted from Huh7 hepatoma, HepG2 hepatoma, and HeLa cells was performed as described under “Materials and Methods.” The blots were hybridized with radiolabeled cDNA probes specific for SNAT2 or L7a (Panel A). Quantification of the Northern blot analysis (Panel B) was performed by scanning densitometry of the film and data assembly as described under “Materials and Methods.” Statistically significant differences are indicated by asterisks, *p < 0.005.

---

www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html.
SNAT2 genes were aligned using the Vector NTI suite version 7 (Informax, Inc., Bethesda, MD). The GenBank™ accession numbers used to obtain the intronic sequences were: mouse, AC109201; rat, NW_044046.1; and human, NT_029419.

RESULTS

SNAT2 Gene Structure Is Similar in Three Mammalian Species—An mRNA to genomic alignment was employed to visualize the SNAT2 gene structures for human, mouse, and rat. In all three species examined, the gene consists of 16 exons and 15 introns, with exon 1 encoding most of the 5′-untranslated region (Fig. 1). The apparent translation start and stop codons are located in exons 2 and 16, respectively.

SNAT2 mRNA Content Is Increased following Amino Acid Deprivation—To determine whether HepG2 hepatoma cells were a suitable model for our study, a comparative analysis of SNAT2 mRNA levels was conducted in three human cell lines: HepG2 hepatoma, Huh7 hepatoma, and HeLa cells. Consistent with previous results, SNAT2 mRNA content was increased in all cell lines examined, with enhancements of 7 to 17 times the control values (Fig. 2). Given the extensive information on System A activity and amino acid control of transcription that has been published in HepG2 cells, these cells were chosen as the model system for the remaining experiments.

Transcription from the SNAT2 Promoter Does Not Change in Response to Amino Acid Deprivation—To identify the genomic sequences necessary for the amino acid-dependent regulation of the human SNAT2 gene, several fragments of its promoter region were tested using firefly luciferase as the reporter. Previous data has shown that luciferase enzymatic activity actually underestimates the amount of transcription in amino acid-deprived cells (32). An asparagine synthetase promoter construct containing nt −115/+1 was used as a positive control (29). The luciferase activities in cells transfected with the human SNAT2 upstream genomic fragments nt −512/+59 or −1111/+59 did not change significantly in response to amino acid removal, whereas the asparagine synthetase promoter construct yielded about a 10-fold increase in the luciferase activity (Fig. 3A). Furthermore, no amino acid-dependent transcription was observed when a longer human SNAT2 genomic fragment (nt −4086/+59) was tested (data not shown). These results suggested that the amino acid response element of the human SNAT2 gene might not reside in the 5′ upstream region. Transfection of two different mouse genomic fragments, nt −1255/+191 or nt −227/+1, also resulted in no detectable amino acid-regulated transcription (Fig. 3B).

A SNAT2 Amino Acid Responsive Element Is Located in the First Intron—To investigate the possibility of an AARE located downstream of the transcription start site, several constructs were prepared from the mouse gene that contained a minimal promoter region (nt −227/+1) as well as downstream genomic sequences through nt +764. These sequences were cloned into the firefly luciferase reporter vector and their ability to drive regulated transcription was measured under histidine-deprived conditions (Fig. 4A). A construct containing the proximal promoter, the entire exon 1, and a large portion of intron 1 (nt −227/+764) exhibited a relatively small, but reproducible increase in transcription following histidine deprivation. Deletion in the 5′ direction yielded two constructs (nt −227/+627, −227/+571) that had significantly less basal transcription, but

**Fig. 3. Transcription from the SNAT2 5′ upstream sequence does not change in response to amino acid deprivation.** HepG2 cells were transiently transfected with firefly luciferase reporter constructs containing the indicated genomic fragments of either the human (Panel A) or mouse (Panel B) SNAT2 5′ upstream sequence. The human asparagine synthetase proximal promoter (Asns [asparagine synthetase] nt −115 to +1) was used as a positive control. Sixteen hours after transfection, the cells were incubated for 10 h in either complete (MEM) medium or medium lacking histidine (−His), harvested, and the luciferase activities were measured as described under “Materials and Methods.” Statistically significant differences are indicated by an asterisk, * p < 0.005.

**Fig. 4. Amino acid responsive elements are located in the first intron of the SNAT2 gene.** HepG2 cells were transiently transfected with luciferase reporter constructs containing the indicated genomic fragments of either the mouse (Panel A) or human (Panel B) SNAT2 gene. Sixteen hours after transfection, the cells were incubated for 10 h in complete (MEM) medium or medium lacking histidine (−His), then lysed and the luciferase activities were measured as described under “Materials and Methods.” Statistically significant differences are indicated by asterisks, *, p < 0.05; **, p < 0.005.
maintained a high rate of transcription following amino acid limitation (Fig. 4A). Further deletion to a fragment containing nt $-222/+467$ resulted in a complete loss of the regulated transcription. When the corresponding regions of the human gene were tested, similar results were obtained (Fig. 4B). The human proximal promoter (nt $-222/-1$) was negative with regard to enhancement of transcription, while including 770 nt of the downstream sequence, in combination with either 218 or 512 nt of the upstream human SNAT2 promoter region, resulted in amino acid-regulated transcription. Deletion of the nt $-512/+770$ fragment to one containing nt $-512/+698$ resulted in loss of the amino acid responsiveness (Fig. 4B). Collectively, the data demonstrate that both the human and mouse SNAT2 genes contain an AARE activity that is located in intron 1 and, for the human gene, the AARE resides within nt $698/770$.

A Specific SNAT2 Intronic Sequence Is Required for Amino Acid-dependent Regulation—Given that the AARE activity of the human SNAT2 gene was contained within a 72-nt sequence, the respective regions from the human, mouse, and rat genomes were aligned (Fig. 5). Three highly conserved regions were identified. Interestingly, sequence 1 (nt $693/701$) includes a CAAT motif and will be called the “CAAT box,” sequence 2 (nt $707/723$) contains a 9-nucleotide core that is identical to the human CHOP AARE and will be termed the “AARE,” and sequence 3 (nt $738/746$) is a purine-rich sequence of 9 nucleotides that will be referred to as the “purine-rich (PuR) box.” The corresponding genomic sequence numbers for the mouse and rat genomes are shown in Fig. 5B. To test whether or not these three conserved sequences are functionally important for amino acid-dependent regulation, multiple mutations within each of these boxes were produced in the context of the human SNAT2 $-512/+770$ genomic fragment (Fig. 6A). The wild-type sequence nt $-512/+770$ yielded an increase of 15 times control in response to histidine limitation (Fig. 6B). Mutagenesis of the CAAT box had no effect on basal transcription, but decreased the induced rate to about 40% of the wild-type levels. Conversely, mutating the AARE-core element almost completely abolished the amino acid regulation. The AARE nucleotides chosen for mutagenesis had been demonstrated to be critical for activity of the human CHOP AARE (26). However, when the PuR box was mutated, no change was observed in the absolute amount of induced transcription, whereas the basal transcription (complete MEM) was 10-fold higher compared with the wild-type (Fig. 6B). Interestingly, although the AARE was absolutely required for the amino acid-deprivation response, it could not sustain the wild-type levels of transcription when the other two sequences were mutated simultaneously. On the other hand, leaving only the CAAT box intact was not sufficient for stimulated reporter gene expression either.

To further characterize the three conserved SNAT2 sequences, the following fragments were placed immediately following the human SNAT2 promoter (nt $-512/+59$) in the luciferase expression vector: nt $+702/+730$, a 29-mer containing the AARE only; nt $+689/+730$, a 42-mer containing the CAAT box and the AARE (CAAT/AARE); and nt $+689/+754$, a 66-mer
containing all three conserved elements. The promoter-only sequence, nt −512/+59, was used as a negative control, and the −512/+770 fragment was used as a positive control (Fig. 7A). As expected, the human SNAT2 promoter region alone did not respond to histidine deprivation, whereas transcription from the −512/+770 construct was increased by more than 10 times the MEM control value and the intronic construct containing nt −512/+770 was set to 1, and all other measurements, both MEM and −His, were recalculated accordingly. Statistically significant differences are indicated by asterisks, *, p < 0.05; **, p < 0.005.

The Human SNAT2 CAAT/AARE Combination Functions as an Enhancer—To test whether or not the CAAT/AARE combination possesses enhancer properties, nt +689/+730 were cloned, in conjunction with the SNAT2 promoter (−512/+59), in two positions: 3' to the transcription start site, but 5' to the firefly luciferase coding sequence (BglII site in the pGL3 plasmid), and 3' relative to the luciferase coding region (BamHI site), in both forward and reverse orientations (Fig. 8A). Mimicking the natural position of the regulatory elements (3' relative to the transcription start site, but 5' to the protein coding sequence, in the forward orientation) yielded the greatest degree of enhancement in response to amino acid limitation.

Fig. 7. Deletion analysis of the human SNAT2 genomic fragment containing SNAT2 nt +689 to +770. HepG2 cells were transiently transfected with the firefly luciferase reporter gene driven by the SNAT2 promoter region (nt −512 to +59) and the following fragments from intron 1: AARE = nt +702/+730; CAAT + AARE = nt +689/+730; all 3 sites = nt +689/+754. The promoter-less firefly reporter vector was used as a negative control. Sixteen hours after transfection the cells were incubated for 10 h in either complete (MEM) medium or medium lacking histidine (−His), then lysed and luciferase activities were measured. Statistically significant differences are indicated by asterisks, *, p < 0.005. In Panel A, the MEM value of luciferase activity for the wild-type construct containing nt −512/+770 was set to 1, and all other values were recalculated accordingly. This graph shows the regulation of the transcription rates in complete (MEM) amino acid medium. In Panel B, the MEM value for each of the individual constructs was set to 1 to better illustrate the 10-fold induction following histidine deprivation.
However, even placement of the amino acid-sensing CAAT/AARE downstream of the luciferase coding sequence permitted a significant stimulation of transcription. Although the absolute rate and the -fold increase were reduced, the presence of the CAAT/AARE in the reverse orientation still resulted in amino acid responsive transcription, regardless of location (Fig. 8A). The CAAT/AARE regulatory sequence was also cloned into the firefly luciferase reporter plasmid driven by the SV40 promoter. The SNAT2 promoter construct −512/+59 was used as a negative control. To obtain the results of Panel B, the SNAT2 CAAT/AARE sequence (nt +689/+730) was cloned in conjunction with the firefly luciferase reporter gene driven by the heterologous SV40 promoter. Once again, the sequence was placed in both forward and reverse orientations (Fwd and Rev, respectively), and in two different positions, the BglII site, which is 3’ to the promoter and 5’ to the luciferase coding region, and the BamHI site, which is 3’ to the luciferase coding region. The non-inducible SNAT2 promoter construct −512/+59 was used as a negative control. To obtain the results of Panel B, the SNAT2 CAAT/AARE sequence (nt +689/+730) was cloned in conjunction with the firefly luciferase reporter gene driven by the heterologous SV40 promoter. Once again, the sequence was placed in both forward and reverse orientations (Fwd and Rev, respectively), and in two different positions, the BglII site, which is 3’ to the promoter and 5’ to the luciferase coding region, and the BamHI site, which is 3’ to the luciferase coding region. The empty pSV40-GL3 luciferase vector was used as the negative control. The -fold increase in response to histidine limitation was obtained by setting each MEM value to 1.0. Statistically significant activation (MEM versus −His for each construct) is indicated by asterisks, * p < 0.005.

Specific DNA-Protein Complexes Are Formed by the AARE—Incubation of a radioactively labeled oligonucleotide, containing the human SNAT2 AARE sequence, with nuclear extracts from HepG2 cells resulted in the formation of three DNA-protein complexes, labeled complexes I-III (Fig. 9, lane 1). Formation of these complexes could be blocked by an excess of unlabeled oligonucleotide (Fig. 9, lane 2), but not by an unrelated sequence (Fig. 9, lane 3). When nuclear extracts from cells incubated in histidine-free medium were tested, the rather diffuse broad bands representing complexes I and III were increased in abundance, whereas the amount of complex II was either unchanged or, in some experiments, slightly reduced (Fig. 9, compare lanes 1 and 5, or 6 and 9). To determine whether all three complexes were associated with the SNAT2 AARE core sequence, a competitor oligonucleotide was prepared that contained the three mutated nucleotides shown in Table II. Although an excess of the unlabeled wild-type oligonucleotide sequence (Spec-wt) blocked formation of the three complexes, when an oligonucleotide containing a mutated AARE core was tested, no competition was observed (Fig. 9, lanes 7 and 10).
Amino Acid Regulation of the SNAT2 Transporter Gene

Fig. 9. Electrophoretic mobility shift analysis of the human SNAT2 AARE sequence. Nuclear extracts were prepared from HepG2 cells maintained in either complete (MEM) or histidine-deficient (-His) MEM for 4 h, as described under “Materials and Methods.” The sequence of the 32P-radiolabeled oligonucleotide probe corresponds to the SNAT2 wild-type 21-mer sequence (nt +706 to +726) and is listed in Table II. Lanes 1–5 and 6–11 represent two independent experiments. Lane 3 (Non-spec) shows the result of the presence of a × 100 excess of an unrelated oligonucleotide (see Table II for sequence), whereas lanes 2, 5, 8, and 11 illustrate the effect of a × 100 excess of the unlabeled probe (Spec). Specific complexes are indicated with the roman numerals I, II, and III. Lanes 6–11 show the results of competition with a × 100 excess of an oligonucleotide comprised of either the wild-type sequence (Spec) or an oligonucleotide containing mutations within the core AARE sequence (Spec-mut). The sequence for the mutated AARE is given in Table II.

Table II

| Name          | Oligonucleotide sequence                          |
|---------------|--------------------------------------------------|
| Unrelated     | GCTTATCGATACCCGAGTCTGGAGTCTCGAATACCTGAGATCT      |
| WT AARE 21mer | ATCGATACCTGAGTCTGGAGTCTCGAATACCTGAGATCT          |
| MUT AARE 21mer| ATCGATACCTGAGTCTGGAGTCTCGAATACCTGAGATCT          |

Discussion

A number of mammalian genes have been identified recently as targets for regulation in response to amino acid availability (reviewed in Refs. 19 and 20). It was first shown in 1972 that System A-mediated amino acid transport activity was increased when cells or tissues were incubated in amino acid limiting medium (6, 7). During the past three decades, many laboratories have contributed to the characterization of this response by following transporter activity and consequently, a large body of information has been accumulated with regard to the characteristics of the response. Following the cloning of three genes encoding System A transport activity, it was documented that the mRNA content for the SNAT2 gene was increased following amino acid deprivation and that this increase paralleled that for System A transporter activity (15–17). The present results provide the first mechanistic information about the substrate-dependent control of SNAT2 transcription by documenting the following novel observations. 1) The data confirm our initial experiments indicating that the increase in SNAT2 mRNA content in HepG2 hepatoma cells is a result of transcriptional activation (17). 2) The results identify the SNAT2 AARE and locate its unique position within intron 1 of the human and mouse genes. 3) Through mutagenesis and deletion analysis it is established that the AARE activity in the SNAT2 gene is enhanced by the presence of a second cis-acting element (CAAT box) located −9 bp upstream of a conserved sequence that contains the AARE core. 4) The results illustrate that the SNAT2 CAAT/AARE sequence has enhancer-like activity in that it conveys amino acid responsiveness to a heterologous promoter, is active in either the forward or reverse orientation, and its function is location independent. 5) The data demonstrate that specific DNA-protein complexes are assembled in vitro on the SNAT2 AARE core sequence and that the abundance of these complexes is increased when nuclear extracts are prepared from amino acid-deprived cells. 6) Computer analysis illustrates that the SNAT2 intronic AARE sequence is highly conserved across species.

A unique feature of the amino acid-dependent regulation of the SNAT2 gene is the intronic location of the AARE. The two genes with the most extensively characterized amino acid responsive elements, human CHOP (25, 26) and human asparagine synthetase (20, 29), have AARE sequences located in the proximal promoter regions. For the asparagine synthetase gene, response to amino acid deprivation requires the presence of two distinct cis-elements, NSRE-1 and NSRE-2, that are separated by 11 bp (29). Although the first of these elements, NSRE-1, differs from the CHOP AARE by only two nucleotides, the CHOP gene does not contain an NSRE-2 sequence. Interestingly, a chimeric construct that places the asparagine synthetase NSRE-2 sequence 11 bp downstream of the CHOP AARE changes the specificity such that this construct now acquires the ability to also respond to the endoplasmic reticulum stress response pathway (26), just like the native asparagine synthetase promoter (24). It will be informative to determine whether the NSRE-2 sequence can also broaden the pathway specificity of the SNAT2 AARE. Another interesting feature regarding the sequence around the AARE in the SNAT2 gene is the presence of the highly conserved sequence containing a CAAT box (5′-TTGACAAT-3′), which is located 9 bp upstream of the conserved region that contains the SNAT2 AARE core. No identical 8-bp sequence is present in the CHOP gene, but interestingly, the central four nucleotides of this element, 5′-GACA-3′, are present upstream of the CHOP AARE at the exact positions and distances that they occupy upstream of the SNAT2 AARE. Whether or not these specific nucleotides play a role in enhancing the amino acid regulation of the CHOP gene, as they do for the SNAT2 gene, remains to be tested.

The present data extend the understanding of System A transporter control by identifying the genomic mechanism for amino acid control of SNAT2 mRNA expression. A thorough characterization of the signal transduction pathways that alter expression from the SNAT2 gene and of the transcriptional machinery that mediates these changes may permit pharmacological modulation of System A activity in selected tissues. The ability to manipulate System A expression is an enticing concept given its likely contribution to the maintenance of elevated growth rates of transformed cells and to the maintenance of hyperglycemia in poorly controlled Type I diabetes.

Acknowledgments—We thank Dr. Jörg Bungert for providing the mouse genomic library and for assistance during library screening. We thank other members of the laboratory for technical advice and helpful discussion.
REFERENCES

1. Bode, B. P. (2001) J. Nutr. 131, 2475S–2485S
2. Broer, S. (2002) Pflugers Arch. 444, 457–466
3. Brosnan, J. T. (2003) J. Nutr. 133, 2068S–2072S
4. Hyde, R., Taylor, P. M., and Hundal, H. S. (2003) Biochem. J. 373, 1–18
5. Saier, M. H., Jr., Daniels, G. A., Boerner, P., and Lin, J. (1988) J. Membr. Biol. 104, 1–20
6. Gazzola, G. C., Franchi, R., Saihene, V., Ronchi, P., and Guidotti, G. G. (1972) Biochim. Biophys. Acta 266, 407–421
7. Riggs, T. R., and Pan, M. W. (1972) Biochem. J. 128, 19–27
8. Tarnuzzer, R. W., Campa, M. J., Qian, N.-X., Englesberg, E., and Kilberg, M. S. (1990) J. Biol. Chem. 265, 13914–13917
9. Varoqui, H., Zhu, H., Yao, D., Mackenzie, B., and Erickson, J. D. (2000) J. Biol. Chem. 275, 4049–4054
10. Yao, D., Mackenzie, B., Ming, H., Varoqui, H., Zhu, H., Hediger, M. A., and Erickson, J. D. (2000) J. Biol. Chem. 275, 22780–22787
11. Sugawara, M., Nakaniishi, T., Fei, Y. J., Huang, W., Ganapathy, M. E., Leibach, F. H., and Ganapathy, V. (2000) J. Biol. Chem. 275, 16473–16477
12. Hatamaka, T., Huang, W., Wang, H., Sugawara, M., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (2000) Biochim. Biophys. Acta 1467, 1–6
13. Sugawara, M., Nakaniishi, T., Fei, Y., Martindale, R. G., Ganapathy, M. E., Leibach, F. H., and Ganapathy, V. (2000) Biochim. Biophys. Acta 1509, 7–13
14. Mackenzie, B., and Erickson, J. D. (2004) Pflugers Arch. in press
15. Gazzola, R. F., Sala, R., Bussolati, O., Visigalli, R., Dall’Asta, V., Ganapathy, V., and Gazzola, G. C. (2001) FEBS Lett. 490, 11–14
16. Ling, R., Bridges, C. C., Sugawara, M., Fujita, T., Leibach, F. H., Prasad, P. D., and Ganapathy, V. (2001) Biochim. Biophys. Acta 1512, 15–21
17. Bain, P. J., LeBlanc-Chaffin, R., Chen, H., Palii, S. S., Leach, K. M., and Kilberg, M. S. (2002) J. Nutr. 132, 3023–3029
18. Jefferson, L. S., and Kimball, S. R. (2003) J. Nutr. 131, 2460S–2466S
19. Lafournoux, P., Bruhat, A., and Jousse, C. (2000) Biochem. J. 351, 1–12
20. Kilberg, M. S., and Barbosa-Tessmann, I. P. (2002) J. Nutr. 132, 1801–1804
21. Gong, S. S., Guerrini, L., and Basilio, C. (1991) Mol. Cell. Biol. 11, 6059–6066
22. Barbosa-Tessmann, I. P., Pineda, V. L., Nick, H. S., Schuster, S. M., and Kilberg, M. S. (1999) Biochem. J. 339, 151–158
23. Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Schuster, S. M., Nick, H. S., and Kilberg, M. S. (1999) J. Biol. Chem. 274, 31139–31144
24. Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Siu, F., Schuster, S. M., Nick, H. S., and Kilberg, M. S. (1999) J. Biol. Chem. 274, 31139–31144
25. Bruhat, A., Jousse, C., Carraro, V., Reimold, A. M., Ferrara, M., and Lafournoux, P. (2000) Mol. Cell. Biol. 20, 7192–7204
26. Bruhat, A., Averous, J., Carraro, V., Zhong, C., Reimold, A. M., Kilberg, M. S., and Lafournoux, P. (2002) J. Biol. Chem. 277, 48107–48114
27. Fawcett, T. W., Martindale, J. L., Guyton, K. Z., Hai, T., and Holbrook, N. J. (1999) Biochem. J. 339, 135–141
28. Wolfgang, C. D., Chen, B. P., Martindale, J. L., Holbrook, N. J., and Hai, T. (1997) Mol. Cell. Biol. 17, 6700–6707
29. Zhong, C., Chen, C., and Kilberg, M. S. (2003) Biochem. J. 372, 603–609
30. Leung-Pineda, V., and Kilberg, M. S. (2002) J. Biol. Chem. 277, 16585–16591
31. Kilberg, M. S. (1989) Methods Enzyme. 173, 564–575
32. Siu, F. Y., Chen, C., Zhong, C., and Kilberg, M. S. (2001) J. Biol. Chem. 276, 48100–48107
