Promoter characterization and role of CRE in the basal transcription of the rat SNAT2 gene

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1Departamento de Fisiología de la Nutrición, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15; 2Posgrado en Ciencias Bioquímicas, Facultad de Química, Universidad Nacional Autónoma de México, and 3Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico

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Ortiz V, Alemán G, Escamilla-Del-Arenal M, Recillas-Targa F, Torres N, Tovar AR. Promoter characterization and role of CRE in the basal transcription of the rat SNAT2 gene. Am J Physiol Endocrinol Metab 300: E1092–E1102, 2011. First published March 8, 2011; doi:10.1152/ajpendo.00459.2010.—Small neutral amino acid transporter 2 (SNAT2) is the most abundant and ubiquitous transporter for zwitterionic short-chain amino acids. The activity of this amino acid transporter is stimulated in vivo or in vitro by glucagon or cAMP analogs. However, it is not known whether the increase in activity at the protein level is due to an increase in SNAT2 gene transcription. Thus, the aim of the present work was to study whether cAMP was able to stimulate SNAT2 gene expression and to localize and characterize the presence of cAMP response elements (CRE) in the promoter that controls the expression of the rat SNAT2 gene. We found that consumption of a high-protein diet that increased serum glucagon concentration or the administration of glucagon or incubation of hepatocytes with forskolin increased the SNAT2 mRNA level. We then isolated the 5′ regulatory region of the SNAT2 gene and determined that the transcriptional start site was located 970 bp upstream of the translation start codon. We identified two potential CRE sites located at −354 and −48 bp. Our results, using deletion analysis of the 5′ regulatory region of the SNAT2 gene, revealed that the CRE site located at −48 bp was fully responsible for SNAT2 regulation by cAMP. This evidence was strongly supported by mutation of the CRE site and EMSA and ChIP analysis. Alignment of rat, mouse, and human sequences revealed that this CRE site is highly conserved among species, indicating its essential role in the regulation of SNAT2 gene expression.

small neutral amino acid transporter 2; small neutral amino acids; cAMP response element; amino acid transport; gene transcription

THE SYSTEM A AMINO ACID TRANSPORTER is ubiquitous in mammalian cells. This sodium-dependent cotransporter works at the cell membrane to actively internalize small neutral amino acids that are utilized in several metabolic processes. It is now known that there are several isoforms of system A named small neutral amino acid transporter (SNAT)1, SNAT2, and SNAT4, which are distributed in a tissue-specific manner (25). However, SNAT2 is present in most tissues, and it possesses all the classic characteristics of system A (34). SNAT2 expression is upregulated in mammalian cells that are incubated in a medium deprived of amino acids, and it is activated by the transcription factors activating transcription factor (ATF) and CCAAT/enhancer-binding protein (29). In addition, system A activity is induced by certain hormones such as insulin, glucagon, and glucocorticoids (3–5, 12, 37). Glucagon, which is released during fasting or after the consumption of a high-protein diet, stimulates the activity of system A to a particularly high degree (4, 12). Glucagon binds to a G protein-coupled receptor located in the plasma membrane that transduces signals by increasing cAMP. An increase in cAMP activates protein kinase A (PKA), which in turn phosphorylates its target proteins, including the cAMP response element-binding protein (CREB). Phosphorylated CREB binds to specific sequences located in the promoter regions of its target genes, which results in activation of their transcription (27). In addition, several studies have shown that incubation of cells with cAMP analogs significantly increases the transport activity of system A, an effect that is repressed by the addition of inhibitors of protein synthesis or transcription (20, 36). The increase in system A activity is associated with an increase in the level of SNAT2 mRNA (18).

The upregulation of SNAT2 gene expression is mediated by several different signal transduction pathways. The current evidence suggests that SNAT2 gene expression is activated by PKA via cAMP and also by PKC, via MAPK, or even by certain hormones such as estradiol (11, 15, 23). These data suggest that the promoter region of the SNAT2 gene has a complex regulatory mechanism that is largely unknown. Currently, it is known that the increase in the transcription of SNAT2 when cells are incubated in a medium deprived of amino acids is regulated by the presence of an amino acid response element along with a conserved CAAT box (29, 30). However, little is known about other molecular mechanisms involved in the regulation of SNAT2 gene transcription.

An increase in the glucagon concentration in proportion to the increase in dietary protein has been demonstrated in rats (35). The increase in glucagon induces the expression of several amino acid-degrading enzymes via cAMP (2, 33). However, it is not known whether SNAT2 expression is modulated by the amount of dietary protein because the amino acids transported by SNAT2 are substrates of some of the amino acid-degrading enzymes. This will result in a concerted mechanism mediated by cAMP between the activity of SNAT2 and the amino acid-degrading enzymes. Thus, understanding the molecular mechanisms that regulate the promoter region of the SNAT2 gene will give clues as to how this amino acid transporter is controlled under different physiological conditions.
In the present study, we demonstrated that expression of SNAT2 is upregulated by the cAMP pathway. In addition, we identified and partially characterized the promoter region of the SNAT2 gene of the rat. With the use of bioinformatics tools, reporter gene assays, band shift, and chromatin immunoprecipitation (ChIP) experiments, we found that the rat SNAT2 gene is transcriptionally regulated through a proximal regulatory cAMP response element (CRE) located −48 bp upstream of the transcription start site by CREB. Because the amino acids transported by SNAT2 are very important for gluconeogenesis, these results are in agreement with previous studies showing that transcription factors of the ATF/CREB family actively participate in the homeostasis of fuel metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**

Switching mechanism at 5′ end of RNA template (SMART) rapid amplification of cDNA end (RACE) cDNA amplification kit was purchased from BD Biosciences-Clontech (Palo Alto, CA). Wizard Genomic DNA purification kit, Luciferase Assay Dual System, Primer Extension system-AMV reverse transcriptase, Gel Shift Assay Systems, pRL-TK Renilla expression vector, and pGL3-basic firefly luciferase vector were from Promega (Madison, WI). The dominant negative (dn-CREB) constructs pCMV-CREB133 (dn-CREB133) and pCMV-KCREB (dn-KCREB) were from Clontech. Restriction endonucleases, DNA ligase, the expanded high-fidelity PCR system, the high pure PCR product purification kit, and Complete Mini protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). RPMI 1640, DMEM, and Lipofectamine 2000 transfection reagent were from Invitrogen-GIBCO (Grand Island, NY). QuickChange site-directed mutagenesis kit was from Stratagene (Cedar Creek, TX). CellLytic Nuclear extraction kit and glucagon were from Sigma (St Louis, MO). Sperm DNA/protein A-agarose 50% slurry was from Upstate Biotechnology (Temecula, CA). Anti-CREB antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of analytical grade.

**Animals and Diets**

**Effect of dietary protein.** Ten male Wistar rats weighing 180 g each were divided into two groups and were housed in stainless steel wired cages. Each group received one of the experimental diets containing 20 or 50% dietary protein for 10 days. Composition of the experimental diets is shown in Table 1. At the end of the study, blood was withdrawn to obtain serum, and a liver sample was immediately frozen in liquid nitrogen and stored at −70°C.

**Effect of the administration of glucagon.** Ten male Wistar rats weighing 180 g had free access to a 10% dietary protein (Table 1) for 5 days. Then, rats were divided into two groups; the experimental group weighing 180 g had free access to a 10% dietary protein (Table 1) for 3 h, livers were removed, and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). This study was approved by the Animal Care Committee at the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran.

**Quantitative Real-Time PCR**

Total RNA was extracted from liver according to Chomczynski and Sacchi (8) and pooled. Three hundred nanograms of total RNA was subjected to quantitative real-time PCR using the TaqMan probe and primer sets for rat SNAT2 (Rn00710421_m1) and β-actin (Rn00667869_ml) and ABI PRISM 7000 system was used for the reaction and detection (Applied Biosystems, Foster City, CA). For each 10-μl TaqMan reaction, 3 μl of total RNA (100 ng/μl) was mixed with 5 μl of One-Step Master Mix, 0.5 μl of Multi-Scribe reverse transcriptase, and 1.5 μl of assay mix containing 200 nM sense and antisense primers and 100 nM TaqMan fluorogenic probe. The PCR scheme used was 48°C for 30 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. β-Actin was used as an invariant control. The relative amounts of all mRNAs were calculated by using the comparative C_T method (User Bulletin no. 2; PE Applied Biosystems).

**Serum Glucagon**

Serum glucagon was assayed with an RIA kit according to the manufacturer (Linco Research Immunobiosays, St. Charles, MO).

**Primer Extension**

To map the transcription start site of rat SNAT2 gene, a primer extension assay was performed using a primer extension kit according to the manufacturer’s instruction. Briefly, an anti-sense primer (see Table 2 for primer sequences) was labeled with [γ-32P]ATP using T4 polynucleotide kinase. The labeled primer was incubated with 10 μg of total RNA or 1 μg of poly(A) RNA from rat tissue for 20 min at 58°C to anneal the primer and RNA and then extended with AMV reverse transcriptase for 30 min at 42°C. The primer extension product was analyzed on a sequencing gel next to the sequencing reaction product.

**RACE**

To confirm the transcription start site of the rat SNAT2 gene, a 5′-RACE was performed using a SMART RACE cDNA amplification kit according to the manufacturer’s instructions. Briefly, 1 μg of total RNA extracted from rat tissue was reverse-transcribed with an oligo(dT) anchor primer and a SMART II A oligonucleotide primer to generate RACE-ready cDNA. Then, 5′-RACE was performed with the universal primer provided in the kit and gene-specific primers (Table 2). The final product was analyzed on a 1.2% agarose-EBBr gel and sequenced.

**In Silico Sequence Analysis**

Human, rat, and mouse genomic DNA sequences were downloaded from the National Center for Biotechnology Information database. The promoter region was predicted by the Neural Network Promoter Prediction program (www.fruitfly.org) and Proscan (www-bimas.cit.nih.gov/molbio/proscan). Transcription factor binding sites were predicted by the MatInspector program (www.genomatix.de) that uses TRANSFAC matrices (vertebrate matrix; core similarity 1.0 and matrix similarity 0.8). Alignments of the mammalian SNAT2 gene structures were generated by using the program Clustal W. The GeneBank accession numbers for SNAT2 sequences were human, NT_029419.11; mouse, NT_039621.7; and rat, NW_047784.1.

**Promoter Cloning and Promoter Deletion Constructs**

The rat SNAT2 putative promoter was amplified by polymerase chain reaction (PCR). Genomic DNA was prepared from rat liver

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**Table 1. Formulation of experimental diets**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Protein, g/kg diet (%)</th>
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<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Casein (90.8% protein)*</td>
<td>110.1</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>364.1</td>
</tr>
<tr>
<td>Dextrose</td>
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</tr>
<tr>
<td>Soybean oil</td>
<td>100.0</td>
</tr>
<tr>
<td>Mineral mix†</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93-VX)‡</td>
<td>10</td>
</tr>
<tr>
<td>Choline citrate</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*“Vitamin-free” casein, Harlan Teklad (Madison, WI) research diets. †Rogers-Harper, Harlan Teklad research diets. ‡AIN-93-VX, Harlan Teklad research diets.
Cell culture, Transient Transfection, and Luciferase Reporter Assays

HepG2 cells were grown in RPMI 1640 supplemented with 8% fetal bovine serum, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a 5% CO2 incubator. HepG2 cells (150,000 cells/well) were plated in 12-well plates 24 h before transfection. Cells were transfected at 80% confluency using Lipofectamine 2000 transfection reagent according to the manufacturer’s manual. Briefly, 0.25 μg of each luciferase reporter gene construct was cotransfected with 0.01 μg of pRL-TK Renilla expression vector (Promega), which was used as an internal control to normalize the transfection efficiency. The effect of dn-CREB was assessed in HepG2 cells cotransfected with 0.25 μg of full-length SNAT2 construct and 0.25 μg of dn-CREB-133 or dn-KCREB. Forty-eight hours after transfection, the cells were lysed to measure the luciferase activity using the Luciferase Assay Dual System, and luminometric measurements were made using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Each transfection was done in triplicate, and three independent experiments were performed for each construct.

Culture Primary Rat Hepatocytes

Rat hepatocytes were isolated by the collagenase perfusion technique and separated from nonparenchymal liver cells and debris by centrifugation (6). Cell viability was assessed by the Trypan blue exclusion test and was always >85%. Cells (65,000/cm2) were plated on treated culture dishes and maintained in DMEM (GIBCO-BRL) supplemented with glucose, 1-glutamine, pyridoxine hydrochloride, sodium pyruvate, and 10% heat-inactivated fetal bovine serum. The medium was refreshed to remove dead cells. Total RNA from rat hepatocytes was obtained by using Trizol reagent.

Site-Directed Mutagenesis

To generate the mutant sequences, a plasmid containing the rat SNAT2 sequence from −872 to +265 was used as a wild-type template. The mutagenesis was performed with the QuikChange site-directed mutagenesis kit. The identity and fidelity of the constructs was verified by sequencing. The mutagenic primers used are presented in Table 2 (the mutated nucleotides are underlined).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts from HepG2 cells were prepared using a nuclear extract kit according to the manufacturer’s instructions. Protein concentration was determined by the Lowry method (24). To generate
double-stranded probes, complementary oligonucleotides (sequences in Table 2) were mixed at a 1:1 molar ratio and heated to 95°C for 5 min and then gradually cooled to room temperature. Probes were labeled with [γ-32P]ATP at the end of oligonucleotide using T4 polynucleotide kinase. DNA mobility shift assays were performed using the gel shift assay system according to the manufacturer’s instructions. Reactions were prepared with 5 μg of HepG2 nuclear extracts, 17.5 fmol of each labeled probe, 0.5–1 μg of poly(dI-dC), and binding buffer. The reactions were incubated for 30 min at room temperature. For competition electrophoretic mobility shift assay (EMSA), 200-fold (3.5 pmol) excess unlabeled double-stranded probe was added to the binding reaction. For super shift analysis, 1 μg of antibody specific for CREB1 was added for 15 min before incubation of the probe with the nuclear extract. Binding reactions were terminated with the addition of gel-loading buffer. The complexes were separated on a nondenaturing 4% (wt/vol) polyacrylamide gel and visualized by autoradiography of the dried gel.

ChIP Assay

Rat primary hepatocytes were isolated as described above, and 5 x 10⁶ cells were grown for 24 h in DMEM containing 10% fetal bovine serum to >90% confluence. Then, cross-linking was performed with 1% formaldehyde for 10 min at room temperature and quenched with 125 mM glycine for 5 min at 37°C. Cells were washed twice with ice-cold phosphate-buffered saline, scraped off the plate, and collected by centrifugation for 5 min at 500 g. The cell pellets were resuspended in 400 μl of SDS lysis buffer (50 mM Tris·HCl, pH 8.1, 10 mM EDTA, 1% SDS) containing a protease inhibitor mixture. Samples were incubated on ice for 10 min and centrifuged at 700 g for 4 min. Pellets were resuspended in 400 μl of Chen’s buffer (CHIP buffer; 150 mM NaCl, 25 mM Tris·HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate) with protease inhibitor mixture and sonicated twice for 30 s each (Braun Biotech International, Melsungen, Germany), followed by centrifugation at 20,000 g at 4°C for 10 min. The supernatants containing the DNA-protein complexes were diluted twofold in ChIP buffer and precleared with 80 μl of salmon sperm DNA-protein A-agarose, 50% slurry (Upstate Biotechnology, Lake Placid, NY), for 30 min at 4°C with rotation. The supernatant was incubated with 4 μg of anti-CREB1 antibody at 4°C overnight. To collect the antibody-protein-DNA complex, 60 μl of protein A-agarose slurry was added for 1 h at 4°C. The protein A-agarose was washed once with 1 ml of each one of the following buffers: low-salt wash buffer (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), high-salt wash buffer (20 mM Tris·HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), and LiCl wash buffer (250 mM LiCl, 20 mM Tris·HCl, pH 8.1, 500 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 2 mM EDTA), followed by two washes with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Complexes were eluted with 250 μl of elution buffer (1% SDS, 100 mM NaHCO₃), and 20 μl of 5 M NaCl was added to reverse cross-link at 65°C overnight. Eluates were incubated with 2 μl of proteinase K (10 mg/ml) for 1 h at 45°C. DNA was recovered by 1 vol of phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in 50 μl of water. A total of 8 μl of purified sample was used for PCR analysis. PCR analysis was performed with specific primers flanking the putative binding sites for CREB in the SNAT2 promoter (pS63-forward and pS-reverse; see Table 2 for sequences) as follows: 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. As a negative control, IgG was used. PCR products were analyzed in a 1.5% agarose gel.

Statistical Analysis

Results obtained in this study are presented as means ± SE. Data were evaluated with Student’s t-test, and it was considered significant at P < 0.05. Data were analyzed with Statview software (Abacus Concepts, San Francisco, CA).

RESULTS

Effect of a High-Protein Diet and Glucagon on SNAT2 Expression

We studied whether the consumption of a high-protein diet increased SNAT2 mRNA in the liver of rats. We found that rats fed a 50% casein diet (high-protein diet) increased SNAT2 mRNA abundance by almost double (P < 0.05) compared with rats fed a 20% casein diet (Fig. 1A). The change in SNAT2 expression was accompanied by a significant increase in serum glucagon in rats fed a 50% casein diet (Fig. 1B). These data suggested that glucagon was able to increase SNAT2 gene expression. To test the effect of glucagon on SNAT2 expression, rats fed a 10% casein diet for 5 days were challenged with an intraperitoneally single dose of glucagon (0.6 mg/kg body wt) (2). This resulted in a significant increase in SNAT2 mRNA abundance compared with rats injected with vehicle (Fig. 1C). The increment in SNAT2 mRNA with glucagon was similar to that observed after the consumption of a high-protein diet. Since glucagon mediates several of its effects through cAMP signaling, we thus incubated primary hepatocytes with forskolin, an inducer of cAMP. Our results showed that forskolin was also able to double SNAT2 mRNA abundance (Fig. 1D), supporting the conclusion that the induction of the SNAT2 gene is mediated via cAMP.

Transcription Start Site of the Rat SNAT2 Gene

To study the role of cAMP on SNAT2 expression, it was necessary to analyze the 5’-regulatory region of the SNAT2 gene. We first had to determine the transcription start site (TSS) of this gene in the rat genome. For this purpose, a primer extension assay was performed using an antisense primer located in the first exon and total RNA from the rat. We detected three bands, indicating the presence of multiple start sites. The upper band was 44 nt and was obtained using either total RNA or RNA poly(A)+. Sequencing reactions with the same primer determined that the TSS is C, corresponding to position 968 bp upstream of the translation start codon (Fig. 2A). Primer extension is a common method to determine the TSS of a gene. However, a frequently encountered problem of this assay is that the reverse transcriptase pauses or terminates at various sites before reaching the 5’ end of the mRNA, which may explain the three bands observed in this study. Thus, to solve this problem, we used a 5’ RACE assay to confirm the TSS. Our results clearly showed that the SNAT2 gene has a single TSS (Fig. 2B). Sequencing of this product demonstrated that the TSS is A, located 970 nt from translation start site, and it corresponded to the TSS predicted by the Neural Network software (Fig. 2C).

Identification of Putative Transcription Factor Binding Sites

We analyzed a fragment of ~1,100 bp with the MatInspector program from Genomatix that included 1,030 bp upstream of the TSS. The sequence contained a TATA box, several potential AP-1, AP-2, and GC box/specificity protein-1 (SP-1) binding sites, several glucocorticoid response elements, and a carbohydrate response element. In addition, we identified sev-
eral sites for different members of the family of Kruppel-like factors in this region as well as two potential CREs located 354 and 48 nt from the TSS (Fig. 3).

Identification of the Transcriptional Regulatory Region of the Rat SNAT2 Gene

Based on the in silico analysis of potential transcription factor binding sites, we proceeded to determine the promoter activity of the 5'-flanking region of the SNAT2 gene. For this purpose, 1,137 bp of the genomic region, containing the first 872 bp upstream of the TSS, was amplified and inserted into the promoterless luciferase reporter plasmid (872/265), and a series of unidirectional 5'-deletion constructs were generated from the total sequence. The constructs were transfected into HepG2 cells, and their luciferase activities were determined. As shown in Fig. 4A, the longest construct (872 to +265) displayed a significantly higher promoter activity in HepG2 cells than that of the promoterless luciferase empty vector pGL3 basic. Deletion up to position 336 resulted in a 25% reduction of promoter activity. In contrast, deletion to 192 resulted in a significant decrease of ~86% of promoter activity. Deletion to 63 resulted in further decrease of promoter activity, indicating that this fragment contains the core promoter, since no promoter activity was observed with smaller fragments (~22 and ~8; Fig. 4A).

Identification of Potential CRE Functional Elements in the SNAT2 Promoter

To identify potential CRE functional elements, we incubated transfected HepG2 cells with the above constructs of the SNAT2 gene with 10 μM forskolin. We observed that this inducer of adenylate cyclase increased promoter activity by ~100%, although it was not different between constructs containing the segments 872 to +265 and 336 to +265. Interestingly, despite the fact that the fragment from 63 to +265 showed a substantial reduction in basal promoter activity, forskolin was still able to increase the activity by 76% (P < 0.05). These data suggest that the CRE site located 48 bp from the TSS is essential for mediating the effects of cAMP on the SNAT2 gene promoter (Fig. 4B). Interestingly, the deletion of the fragment 336 to 192 bp significantly reduced basal promoter activity, suggesting the presence of a positive regulatory site in this region; however, more studies are needed to determine the elements involved in this response.

Site-Directed Mutagenesis of CRE Sites in the SNAT2 Gene Promoter

To further assess the conclusion that the CRE site located at ~48 bp is essential for regulating the effects of cAMP on the SNAT2 promoter, we performed site-directed mutagenesis in the two CRE sites located at ~354 and ~48 bp from TSS (Fig. 5A). Our results clearly demonstrated that mutation of CRE at ~354 bp did not have any effect on the response to forskolin; however, mutation of CRE at ~48 bp dramatically reduced the response to forskolin, supporting the evidence that this binding site is essential for the regulation of SNAT2 gene expression by cAMP (Fig. 5B). Mutation of both sites did further reduce the response to forskolin, indicating that the CRE site at ~48 bp is essential for mediating cAMP effect (Fig. 5B).

Fig. 1. Expression of small neutral amino acid transporter 2 (SNAT2) in the liver of rats fed a high-protein diet or injected with glucagon or in primary rat hepatocytes incubated with forskolin. A: effect of dietary protein concentration on the abundance of SNAT2 mRNA. Rats were fed a 20 or 50% casein diet for 10 days. B: serum glucagon concentration in rats fed a 20 or 50% casein diet for 10 days. C: effect of intraperitoneal glucagon injection on the abundance of SNAT2 mRNA. Rats were injected with 0.6 mg/kg body wt, and 3 h later SNAT2 mRNA was measured. D: effect of forskolin on the abundance of SNAT2 mRNA. Primary hepatocytes were incubated with 10 μM forskolin for 4 h. Values are means ± SE; n = 5 rats/group. *Significant differences (P < 0.05).
CREB Binding to the SNAT2 CRE Site at −48 bp in Vitro

Because it is known that the cAMP signaling pathway involves the activation of the transcription factor CREB, we conducted EMSA to assess whether the CRE sites were able to bind CREB. As shown in Fig. 6A, lanes 3 and 5, both CRE sites at −48 and −354 bp produced retarded bands with HepG2 nuclear extracts. However, mutation of CRE site at −354 bp (Fig. 6A, lane 4) enhanced the formation of the complex, indicating an unspecific binding, whereas mutation of CRE at −48 bp (Fig. 6A, lane 6) abolished the formation of the complex, suggesting that this binding site binds CREB. To further investigate the specificity of the binding, a competition assay was performed. As shown in Fig. 6B, lanes 6 and 7, specificity of the binding at the −48-bp CRE site was confirmed by competition with 200-fold molar excess of wild-type CRE binding site consensus sequence (31). As a positive control, we used the CRE consensus sequence (Fig. 6B, lane 2), which showed a very intense retarded band that was competitively inhibited by the addition of an excess of unlabeled consensus CRE (Fig. 6B, lane 3). To identify whether CREB was bound to the CRE sequence, we performed a supershift assay using an anti-CREB1 antibody. Figure 6C, lane 6, clearly demonstrates that preincubation with the antibody decreased the intensity of the retarded band for the CRE site at −48 bp, similar to the consensus sequence (Fig. 6C, lane 5), indicating that CREB was indeed bound to this sequence.

CREB Binding to the SNAT2 CRE Site at −48 bp in HepG2 Cells

To demonstrate whether CREB was bound to the SNAT2 CRE site at −48 bp, intact living HepG2 cells were stimulated with forskolin, and a ChIP assay was performed at different time points. A small signal was obtained at 0 and 30 min, indicating basal CRE binding to the CRE site at −48 bp (Fig. 7A). Interestingly, we observed an intense amplification of the signal after 60 min of incubation with forskolin that was sustained until 120 min (Fig. 7, A and B). These data clearly demonstrate that CREB recognized and bound actively to the SNAT2 CRE site at −48 bp, supporting the role of CREB as an inducer of SNAT2 gene expression. No signal was observed with DNA recovered from IgG.

Effect of dn-CREB on SNAT2 Reporter Activity

To further demonstrate whether CREB is involved in the SNAT2 transcription regulation, we studied whether inhibition of CREB would reduce SNAT2 promoter activity in HepG2 cells. As expected, forskolin increased the promoter activity of the full-length construct by 100% with respect to unstimulated cells. Addition of either dn-CREB-133 or dn-KCREB significantly prevented the stimulation of forskolin compared with cells containing only the full-length construct, indicating that CREB was necessary to stimulate SNAT2 gene transcription via cAMP (Fig. 8).

The CRE Site at −48 bp is Conserved in the Genomic Sequence of Different Species

To identify whether the CRE site at −48 bp in the rat SNAT2 gene was also present in other species, we performed an alignment of the genomic sequences of the mouse and human SNAT2 genes with the rat SNAT2 gene. We identified two conserved sequences in the proximal promoter, a TATA box (nt −29 to −24) and the CRE motif (nt −48 to −41). In each of the three sequences, the CRE motif is a half-site motif (ATGAC) of the full CRE palindrome (32) (Fig. 9). These results suggest that regulation of the SNAT2 gene by CREB is a conserved mechanism among species.
DISCUSSION

SNAT2 is an amino acid transporter that plays an essential role in the formation of the amino acid pool of the cell (7). Therefore, understanding the different mechanisms that regulate the expression of this transporter can provide clues as to how SNAT2 maintains cellular homeostasis under different physiological circumstances. Our results clearly showed that feeding rats a high-protein diet increased the expression of SNAT2, suggesting that this transporter plays an important role in providing amino acids to the oxidative pathways for amino acid degradation, allowing the cell to remove the excess of amino acids supplied by the diet. In addition, it has been demonstrated that SNAT2 may play a role in sensing and transporting the amino acids supplied by the diet, thereby activating several signaling pathways to maintain cellular homeostasis (19, 20). Thus, an increase in the expression of SNAT2 by a high-protein diet can amplify all of these responses.

Our results showed that consumption of a high-protein diet increased serum glucagon concentration, and we demonstrated that a glucagon injection increased SNAT2 mRNA abundance. Glucagon effects are mediated by activation of adenylate cyclase. We observed that cells incubated with forskolin, an activator of adenylate cyclase, also stimulated the expression of the SNAT2 gene. These results suggest that the increase in the expression of SNAT2 is mediated via cAMP. To confirm this hypothesis, we studied the promoter region of the SNAT2 gene with the aim of finding potential response elements activated by cAMP.

For this purpose, we mapped the transcription start site of the SNAT2 gene in the rat. Our results showed that it has a single transcription start site localized 970 bp from the ATG codon that initiates translation. In addition, it has been demonstrated that SNAT2 may play a role in sensing and transporting the amino acids supplied by the diet, thereby activating several signaling pathways to maintain cellular homeostasis (19, 20). Thus, an increase in the expression of SNAT2 by a high-protein diet can amplify all of these responses.
sequence (TGACGTCA) (27). However, it has been demonstrated that differential regulation of target genes by cAMP through CREB is achieved by varying the sequence composition and placement of the CRE site within the promoter. It is known that a half-site motif (ATGAC) is sufficient to promote transcription of target genes, although it is less active than the full CRE palindrome (9). In addition, it has been reported that half-site motif sequences can also bind other transcription factors such as AP-1, ATF, or heterodimers with c-Fos or c-Jun, thereby allowing cross-talk between CREB and other transcrip-

**Fig. 4. Functional analysis of the rat SNAT2 gene promoter.** Fragments of the indicated sizes in the 5' region of the rat SNAT2 gene were cloned in the pGL3 basic vector and transfected into HepG2 cells. Relative luciferase (Luc) activity was measured 48 h after transfection. A: the basal promoter activity of the SNAT2 gene. B: effect of 10 μM forskolin on SNAT2 promoter activity. HepG2 cells were cotransfected with each promoter-reporter construct and pRL-TK and incubated in presence or absence of forskolin. Luc activities were measured 48 h after transfection. The results are means ± SE of 3 independent experiments per construct. *P < 0.05, **P < 0.01; significant difference vs. the respective control.

**Fig. 5. Mutation analysis of the CRE binding sites in the SNAT2 promoter.** A: oligonucleotide sequences containing wild-type or mutated CRE sites of SNAT2. B: Luc activity of constructs with mutations of the CRE sites at -48 and/or at -354. HepG2 cells were transfected with a Luc reporter plasmid containing wild-type or mutated CRE sites and incubated with or without 10 μM forskolin. Luc activities were measured 48 h after transfection and normalized to Renilla expression. The results are means ± SE of 3 independent experiments per construct. *P < 0.05, significant difference vs. the respective control.
tion factors, which allows for fine-tuning of the regulatory events (26) involved in the transcription of the SNAT2 gene.

It has been demonstrated that the presence of several CRE sites in a promoter can synergistically potentiate the expression of target genes activated by CREB (14, 17, 22). However, this was not the case for the SNAT2 gene, because the CRE site located at −354 bp was nonfunctional according to reporter assays and EMSAs. Interestingly, we clearly established the presence of a functional cAMP response element located −48 bp from the transcription start site. This response element was located in the core promoter, and its mutation significantly reduced basal promoter activity, indicating the essential role of this CRE site in the expression of SNAT2. This result is in agreement with previous studies in human and rat that reported

![Fig. 6. Binding of cAMP response element-binding protein (CREB) to CRE motifs in the SNAT2 promoter in vitro. Double-stranded DNA oligonucleotides containing the CRE consensus sequence (CRE cons), the SNAT2 CRE site at nt −48, and the SNAT2 CRE site at −354 were incubated with HepG2 nuclear extracts. The DNA sequences of the oligonucleotides used are indicated in Table 2. A: electrophoretic mobility shift assay analysis of HepG2 nuclear extracts with labeled oligonucleotides containing the CRE consensus site (lane 2), the wild-type CRE site at −354 and −48 bp (lanes 3 and 5), and mutated CRE sites at −354 (CRE354m) and −48 bp (CRE48m) (lanes 4 and 6). B: competitive assays with an excess of unlabeled CRE consensus sequence with CRE consensus, CRE −354 site, and CRE −48 site are shown in lanes 3, 5, and 7, respectively. C: supershift assay was performed by preincubating 1 μg of antibody anti-CREB1 with HepG2 nuclear extracts. Probes for the labeled consensus CRE sequence (lanes 2 and 5), the CRE site at −48 (lanes 3 and 6), and the CRE site at −354 (lanes 4 and 7) were added. Lane 1 in each panel corresponds to free probe (FP) as a negative control. The binding reactions were separated using a 4% nondenaturing polyacrylamide gels. SC, specific competitor (unlabeled consensus).](http://ajpendo.physiology.org/)
that most CRE elements are located adjacent to the transcription start site and have downstream TATA boxes necessary for optimal induction in response to cAMP (21, 39). Nonetheless, binding to the CRE site located at /H11002 48 bp was less intense than that observed with the consensus sequence, as determined by EMSA. As indicated above, this is possibly because this element was one-half of the full CRE palindrome sequence (9, 38), however, its functional activity was clearly demonstrated by the reporter and ChIP assays.

We also studied the kinetics of activation of SNAT2 after the incubation of primary hepatocytes with forskolin. We observed significant immunoprecipitation of the CRE-CREB complex after 60 min of incubation with forskolin, which was preserved until 120 min. This is in agreement with previous evidence showing that the transcription of cellular genes usually peaks 30 min after stimulation with cAMP, coinciding with the time required for levels of the C subunit of PKA to reach maximal levels in the nucleus and the fact that CREB activity attenuates after 2–4 h (16, 27).

In addition, our study showed that phosphorylation of CREB is necessary to induce SNAT2 gene transcription since dn-CREB-133 containing a mutation in Ser133, a residue that is phosphorylated by PKA to induce CREB activity (27), prevented promoter activity stimulated by forskolin. Furthermore, as we observed in the EMSA and ChIP analysis, binding of CREB to its response element site is a requirement for the stimulation of the SNAT2 gene promoter activity since dn-KCREB that has a mutation in the DNA-binding domain also reduced the activity of the reporter assay.

The sequence alignment of the SNAT2 gene in rat, mouse, and human revealed that the apparent transcription start site, the TATA box, and the CRE site are highly conserved, suggesting that the mechanism of regulation of the SNAT2 gene by cAMP is similar in these species. In fact, an increase in SNAT2 activity/expression by stimulation with glucagon, activators, or analogs of cAMP has been demonstrated in rat cells (4, 10); however, it was not observed in placental BeWo cells (28). Interestingly, promoter regions of amino acid-degrading enzymes also possess CRE sites (1, 13, 33), suggesting that activation of transcription via cAMP can synchronically modulate amino acid metabolism under different metabolic conditions, such as fasting or high proteolytic wasting diseases, or after the consumption of diets containing excess protein.

In addition to the CRE element, we also found potential binding sites for several transcription factors such as SP-1, AP-1, glucocorticoid response element, carbohydrate response element, and GC boxes. However, more studies are needed to identify whether these sites have any functional significance for the expression of SNAT2.

This is the first report that investigates the molecular mechanism by which cAMP activates the expression of the SNAT2 gene. Although it has been established by several studies that glucagon or cAMP analogs are able to stimulate the activity of system A, we have now identified, in the promoter region of the SNAT2 gene, the key response element that upregulates the expression of this transporter for small neutral amino acids by cAMP.

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