The Mouse Na\(^+\)-Sulfate Cotransporter Gene *Na\(\text{s}\)1*

CLONING, TISSUE DISTRIBUTION, GENE STRUCTURE, CHROMOSOMAL ASSIGNMENT, AND TRANSCRIPTIONAL REGULATION BY VITAMIN D\(*\)

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Na\(\text{s}\)1 is a Na\(^+\)-sulfate cotransporter expressed on the apical membrane of the renal proximal tubule and plays an important role in sulfate reabsorption. To understand the molecular mechanisms that mediate the regulation of Na\(\text{s}\)1, we have isolated and characterized the mouse Na\(\text{s}\)1 cDNA (mNa\(\text{s}\)1-1), gene (Na\(\text{s}\)1), and promoter region and determined Na\(\text{s}\)1 chromosomal localization. The mNa\(\text{s}\)1-1 cDNA encodes a protein of 594 amino acids with 13 putative transmembrane segments, inducing high affinity Na\(^+\)-dependent transport of sulfate in *Xenopus* oocytes. Three different mNa\(\text{s}\)1-1 transcripts derived from alternative polyadenylation and splicing were identified in kidney and intestine. The Na\(\text{s}\)1 gene is a single copy gene comprising 15 exons spread over 75 kilobase pairs that maps to mouse chromosome 6. Transcription initiation occurs from a single site, 29 base pairs downstream to a TATA box-like sequence. The promoter is AT-rich (61%), contains a number of well characterized cis-acting elements, and can drive basal transcriptional activity in opossum kidney cells but not in COS-1 or NIH3T3 cells. We demonstrated that 1,25-dihydroxyvitamin D\(_3\) stimulated the transcription of the Na\(\text{s}\)1 promoter in transiently transfected opossum kidney cells. This study represents the first characterization of the genomic organization of a Na\(^+\)-sulfate cotransporter gene. It also provides the basis for a detailed analysis of Na\(\text{s}\)1 gene regulation and the tools required for assessing Na\(\text{s}\)1 role in sulfate homeostasis using targeted gene manipulation in mice.

Sulfate is the fourth most abundant anion in mammalian plasma, is present in nearly all cell types, and is essential for a variety of metabolic and cellular processes (1). The largest group of sulfate conjugates in mammals is sulfated proteoglycans, which are required for normal structure and function of bone and cartilage. Accordingly, three human congenital chondrodysplasias were recently shown to be caused by mutations in a sulfate transport protein gene (DTDST),\(^1\) leading to undersulfation of proteoglycans in the extracellular matrix of bone and cartilage, and associated developmental abnormalities (2–4). Considering the importance of sulfate at a cellular and biochemical level, it is likely that mechanisms regulating the serum sulfate levels are essential for the maintenance of normal physiology. However, little is established about the molecular factors that regulate sulfate homeostasis, and the physiological consequence of a disturbance in sulfate homeostasis is mostly unknown.

In mammals, the regulation of sulfate homeostasis is largely determined by the kidney with the majority of the filtered sulfate load reabsorbed in the proximal segment of the nephron. Transepithelial transport of sulfate from the renal lumen to the blood compartment involves entry through the brush-border membrane (BBM) by a Na\(^+\)-dependent transport system, translocation across the cell and efflux across the basolateral membrane by an anion exchange system (5). Early transport studies in BBM vesicles suggested that Na\(^+\)-sulfate cotransport across the BBM is the rate-limiting step in the overall sulfate reabsorptive process (6, 7). By expression cloning, we isolated a cDNA (Na\(\text{s}\)1-1) from rat kidney encoding a high affinity Na\(^+\)-dependent sulfate transporter (8). Na\(\text{s}\)1 mRNA is expressed in kidney and small intestine and encodes a glycosylated protein (8) that has been localized by immunohistochemistry to the BBM of proximal tubular cells (9).

Recently, factors known to regulate renal Na\(^+\)-sulfate reabsorption were found to regulate Na\(\text{s}\)1 expression in the kidney. Vitamin D was shown to modulate concomitantly serum sulfate concentration, renal sulfate handling, and the expression (mRNA and protein levels) and activity of the Na\(\text{s}\)1 cotransporter (10). High sulfate intake in rats led to a reduction in both Na\(\text{s}\)1 mRNA and protein (11), whereas low sulfate intake (reduced methionine diet) led to an increase in both Na\(\text{s}\)1 mRNA and protein (12). Thyroid hormone, growth hormone, heavy metals, potassium intake, and anti-inflammatory agents were also found to regulate Na\(\text{s}\)1 expression (13–17). It is suggested that these modulators could alter serum sulfate levels via the regulation of Na\(\text{s}\)1 expression in vivo, suggesting that sulfate homeostasis is controlled, at least in part, by Na\(\text{s}\)1. However, the underlying mechanisms involved in the regulation of Na\(\text{s}\)1 expression by these factors, as well as Na\(\text{s}\)1 contribution to body sulfate homeostasis, have yet to be defined.

\(^{\text{1}}\) The abbreviations used are: DTDST, diastrophic dysplasia sulfate transporter; BBM, brush-border membrane; RACE, rapid amplification of cDNA ends; UTR, untranslated region; OK, opossum kidney; RT-PCR, reverse transcriptase-polymerase chain reaction; LA-PCR, long and accurate PCR; 1,25-(OH)\(_2\)D\(_3\), 1α,25-dihydroxyvitamin D\(_3\); DR, direct repeat; VDR, vitamin D receptor; VDRE, vitamin D-responsive element; hRXR\(\alpha\), human retinoid X receptor \(\alpha\); TRE, thyroid hormone-responsive elements; GRE, glucocorticoid-responsive element; EST, expressed sequence tag; bp, base pair; kb, kilobase pair; PIPES, 1,4-piperazinediethanesulfonic acid.
In order to provide insights into the molecular mechanisms underlying tissue-specific and hormonal regulation of NaSi-1 and its role in sulfate homeostasis, we have cloned and characterized the mouse NaSi-1 cDNA and its corresponding gene. This study represents the first characterization of the genomic structure of a Na \(^+\)-coupled sulfate transporter gene. We have also determined the pattern of NaSi-1 expression in mouse adult tissues, identified the existence of alternative transcripts, determined its chromosomal localization, and demonstrated that the transcriptional activity of the promoter region is elevated in response to 1,25-(OH)\(_2\)D\(_3\) stimulation in a transiently transfected renal cell line.

**EXPERIMENTAL PROCEDURES**

**PCR Amplifications and Sequencing—Oligonucleotides**

Oligonucleotides used during this study are listed in Table I. The mouse NaSi-1 (designated mNaSi-1) cDNA coding sequence was cloned using RT-PCR. Total RNA (5 \(\mu\)g) isolated from mouse kidney cortex was reverse-transcribed and PCR-amplified using primers derived from the rat NaSi-1 cDNA (designated rNaSi-1) coding sequence (8). The PCR products were subcloned into pCR 2.1 vector (Invitrogen) and sequenced in both directions. Semi-nested PCR was also performed from total RNA treated with the 5′-actin). Dye-termination sequencing was performed using the Big DyeTM Terminator kit (Perkin-Elmer) following the manufacturer’s instructions. Sequencing reactions were carried out using the Applied Biosystems 373 A DNA sequencer (Perkin-Elmer). Sequencing reactions were analyzed using the Sequence Analysis software (Perkin-Elmer).

**Northern and Southern Blot Analyses**

The Northern and Southern blot analyses were performed as previously described (8). Fragments of total RNA were transferred to nylon membranes and hybridized with a full-length32P-labeled mNaSi-1 cDNA probe in Church’s buffer (0.5M Na2HPO4/NaH2PO4, 40 mM EDTA, pH 7.2, 7% SDS, 100 mM NaCl) for 16–18 h at 65 °C. The membranes were washed in 2× SSC, 0.1% SDS at room temperature (RT), then 1× SSC, 0.1% SDS at RT, and finally 0.1× SSC, 0.1% SDS at RT.

**DNA and RNA microinjection**

DNA was microinjected into stage V and VI oocytes using a Nanoject automatic oocyte injector (Drummond Scientific Co.). mNaSi-1 cDNA (5 ng) was co-injected with 50 nl of an actin cRNA using the Nanoject automatic oocyte injector (Drummond Scientific Co.).

**Tissue preparation**

Mouse tissues were homogenized in TRIZOL reagent (Life Technologies, Inc.) and RNA was isolated according to the manufacturer’s instructions.

**RESULTS**

**RNA Isolation and Analysis**

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Cells were then harvested in cell lysis buffer, and the lysate was transfected using LipofectAMINE 2000 reagent (Life Technologies, 10% (v/v) fetal bovine serum (Life Technologies, Inc.). OK cells were in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with -Nas1 promoter and 3229, were then inserted upstream of a luciferase reporter gene in a promoterless-expression vector (pGL3-Basic, Promega) by restriction enzyme digestion and ligation. Plasmids were designated pNas1-3229, pNas1-1203, and pNas1-457, respectively. The 1203-bp promoter fragment was also cloned in reverse orientation and designated pNas1-1203R. Correct insertion and sequence were verified by enzyme restriction digestion and sequencing. COS-1 and NIH3T3 cells were cultured in Dulbecco's modified Eagle’s medium (Life Technologies, Inc.) with 10% (v/v) fetal bovine serum (Life Technologies, Inc.). OK cells were maintained in Ham's F12/Dulbecco's modified Eagle’s medium (1:1) containing 10% fetal bovine serum. At 80% confluence, cells were cotransfected using LipofectAMINE (Life Technologies, Inc.) and with 0.8 μg of the Nas1 gene promoter-luciferase reporter plasmid and 0.2 μg of a control plasmid (pGL3-Basic, Promega) as an internal control for transfection efficiency. Incubation with plasmids and LipofectAMINE was carried out for 24 h in normal growth medium, as recommended by the manufacturer. Controls were performed by transfection with pGL3-Basic (promoter-less plasmid) and pGL3-Control (containing the SV40 promoter). In experiments involving vitamin D, cells were cotransfected with 0.2 μg of a vitamin D receptor expression vector (VDR/pSG5 and RXR/pSG5 plasmids, respectively; a generous gift from Dr. John White, McGill University). The VDR and hRXRα proteins (Fig. 1A) contains one potential protein kinase A (Thr404) and five potential kinase A (Ser213, Thr218, Ser230, Thr322, and Thr322) phosphorylation sites (Fig. 1A). Consensus sequences for N-glycosylation were found at Asn positions 140, 174, and 590 (Fig. 1A). Alignment of the mouse and rat Nas1 mRNA sequence shows 93.6% identity and 96% similarity. Nucleotide sequence identity is 91% between mouse and rat Nas1. When this work was initiated, no ESTs with homology to mNas1-1 were identified. At the submission of this manuscript, a search in the EST data base identified approximately 50 murine ESTs from kidney, all identical to mNas1-1. Homology searches using BLAST (24) and PSI-BLAST (25) revealed significant homology to 22 other proteins (Fig. 1B), although the closest relatives are the recently reported human Na+–sulfate cotransporter SUT-1 (49% identity (26)) and the Na+–dicarboxylate transporters sharing ~32–43% protein sequence identity with mNas1-1. Of particular interest is a consensus pattern previously established for Na+-coupled symporters (PROSITE PS01271) known as the Na+-sulfate signature, present at amino acids 522–538 in the mNas1-1 protein containing a very high degree of homology with other related proteins (Fig. 1B).

To determine the functionality of the isolated clone, we injected mNas1-1 cRNA into Xenopus oocytes followed by [35S]sulfate radiotracer uptake assay. Sulfate uptake in mNas1-1-cRNA-injected oocytes was Na+–dependent and showed typical Michaelis-Menten saturation, with a calculated Km value for sulfate of 0.20 ± 0.06 μM and Vmax of 49.2 ± 4.1 pmol/h (data not shown), in agreement with the BBM Na+–sulfate cotransporter (5). mNas1-1 mRNA expression was screened by RT-PCR in 23 murine tissues (Fig. 2A). An amplified mNas1-1 fragment was obtained in RNA from kidney, duodenum/jejunum, ileum, and colon. Lower levels of mNas1-1 mRNA expression were observed in cecum, testis, adrenal, and adipose tissue. By normalizing the mNas1-1 mRNA signal to β-actin, the relative abundance of mNas1-1 in kidney and ileum was found to be similar and approximately twice as high as those found in duodenum/jejunum and colon (Fig. 2B; n = 3). The physiological importance of the low level of expression of mNas1-1 found in testis, adrenal, and adipose tissue remains to be determined.

Genomic Organization of the Mouse Na+-Sulfate Cotransporter Gene, Nas1—Southern blotting was used to estimate the size and complexity of the gene encoding mNas1-1, designated Nas1 (Fig. 3). Results show that the estimated size of the Nas1 gene was approximately 45 kb, which was lower than the actual size of the Nas1 gene determined from genomic cloning (see below). This was due both to the presence of comigrating bands and large introns that did not hybridize with the cDNA probe. Blots washed at both high and low stringency gave similar results, suggesting that Nas1 is a single copy gene.

Screening of a genomic λ phage library led to the isolation of five λ Nas1 clones containing the 5′-flanking region and most of the Nas1-coding region (Fig. 4A). Introns 1, 2, and 6–8 were present in the λ clones were obtained using LA-PCR (Fig. 4A). The λ clones and PCR-amplified introns overlapped, covering a 80-kb region comprising the entire Nas1 gene (Fig. 4A). Southern analysis of the Nas1 genomic clones was consistent with the data obtained from Southern blotting of mouse genomic DNA and confirmed that Nas1 is a single copy gene. The resulting exon-intron organization of the mouse Nas1 gene is shown in Fig. 4B. The Nas1 spans ~75 kb and contains 15 exons. The translation initiation site is present in exon 1. Exon sizes range from 49 to 188 bp, except for exon 15, which is 555 bp and contains the TGA stop codon (Fig. 4C and Table II). Introns sizes range from 70 bp to 15 kb (Table II). All exon-intron boundaries conform to canonical splice donor and acceptor consensus sequences, and the codon phase usage is mainly

**RESULTS**

**Mouse NaSi-1 cDNA Cloning and Expression**—The mouse Na+-sulfate cotransporter cDNA, mNaSi-1, was cloned using a combination of RT-PCR and 5′- and 3′-RACE techniques. The mNaSi-1 cDNA is 2246 bp long, with 25 bases of 5′UTR, 818 bases of an open reading frame of 1782 bases, and 436 bases of 3′UTR. The 3′UTR terminates at a polyadenylation signal (AATAAA) at position 2180. The open reading frame encodes a protein of 594 amino acids (Fig. 1A) with a calculated molecular mass of 66.1 kDa, containing 13 putative transmembrane domains, predicted by the TopPred2 program (23). The mNaSi-1 protein contains one potential protein kinase A (Thr404) and five potential protein kinase C (Ser213, Thr218, Ser230, Thr322, and
Comparison of predicted protein transmembrane domains to exon border structure showed that each predicted transmembrane segment is encoded by a separate exon, with the exception of transmembrane domains 10 and 11, which are encoded by the same exon (exon 13). In addition, splicing mostly occurred near membrane/aqueous transitions (Fig. 4D).

Mapping of Nas1 to Mouse Chromosome 6—Nas1 was mapped by analysis of the data from the T-31 Radiation Hybrid panel in The Jackson Laboratory Mouse Radiation Hybrid data base. The data placed Nas1 on mouse chromosome 6 in the most likely position between marker D6Mit170 (LOD score 20.8) and D6Mit380 (LOD score 11.2). Nas1 is 2.3 centi-rays distal to D6Mit170, which has been assigned map positions of 4.4 centimorgans (MIT) and 4.0 centimorgans (MGD and Chromosome Committee). The Nas1 gene maps very close to the calcitonin receptor gene, which has been assigned a position of 4.5 centimorgans (MGD and Chromosome Committee) in...
Fig. 2. Tissue distribution of mouse Na\textsuperscript{+}-sulfate cotransporter. A, total RNA from various mouse tissues (indicated at the top) was reverse-transcribed using an oligo(dT) primer and PCR-amplified using mNaSi-1 (FN252 and RN1220, upper bands) and \( \beta \)-actin (lower bands) primers. An aliquot of each PCR reaction was electrophoresed on 1.2% agarose gels and visualized with ethidium bromide. A water blank is shown. B, densitometric analysis of mNaSi-1 RT-PCR-amplified mRNA derived from three separate experiments. Data are shown as mean \( \pm \) S.D.

mouse and 7q21.3-q21.3 in human.

Alternative Polyadenylation and Splicing of the Nas1 Gene—By using Northern blot analysis, two major transcripts of equal intensity (2.2 and 2.5 kb) were detected in kidney, ileum, duodenum/jejunum, and colon but not in liver (Fig. 5A), confirming the RT-PCR data (Fig. 2). Normalizing the mNaSi-1 mRNA signal with \( \beta \)-actin showed that renal and ileal mNaSi-1 transcripts were of similar abundance, in agreement with RT-PCR data (Fig. 2). Whereas the 2.2-kb transcript most probably corresponds to the 2246-bp cDNA fragment characterized above, the larger transcript is most likely derived from alternative polyadenylation, as previously shown for the rNaSi-1 transcripts (8, 27). To test this, we performed 3‘-RACE on total RNA from mouse kidney (Fig. 5B). Two bands were obtained using primers FN110, FN823, and FN1547 (Fig. 5B, lanes 2–4). Sequencing analysis showed that the smaller band corresponded to the 2246-bp mNaSi-1 cDNA, whereas the larger band contained an additional 254 nucleotides at the 3‘ end, generating a 2500-bp cDNA fragment containing a polyadenylation signal at position 2437. Sequence comparison between the 2.5-kb clone and the genomic \( \lambda \)3 clone showed absence of introns in the 3‘-UTR. In addition to the 2.2- and 2.5-kb clones, we could also amplify an additional faint band at 2.1 kb using a sense primer further upstream (Fig. 5B, lane 1). This fragment was 100% identical to the 2.2-kb cDNA, with the exception of a missing 129-bp region, corresponding to exon 2 sequence (Fig. 5C). To determine the functional identity of these clones, we injected the corresponding cRNAs into Xenopus oocytes. Both the 2.2- and 2.5-kb clones induced significant Na\textsuperscript{+}-sulfate cotransport (at a similar rate), whereas the 2.1-kb clone showed an activity comparable to water-injected oocytes (Fig. 5D).

Transcription Initiation Site and Nucleotide Sequence of the Nas1 5‘-Flanking Region—To identify the transcription start site, primer extension assays were performed. A single major product of 70 and 114-nucleotides was identified using primers RN44 and RN88, respectively (Fig. 6A). This located the transcription start site, designated +1, at 26 bp upstream from the translation initiation ATG codon (Fig. 6C). To confirm these data, we performed 5‘-RACE, which gave rise to a 277-bp band (Fig. 6B). Sequence analysis of this fragment confirmed the primer extension findings, locating the transcription start site 28-bp upstream from the ATG codon (Fig. 6C).

A 3229-bp region of the Nas1 promoter has been isolated and sequenced (Fig. 7). This region was found to be A + T-rich (61%). The center of an atypical TATA sequence, TATTTAA, is located 29 bp upstream of the transcription start site. A classical CAAT box consensus sequence is present at position −91 on the negative strand. However, a canonical TATA box and CAAT box are positioned further upstream at −201 and −424, respectively, but are considered too far from the transcription start site to have promoter function. No GC box motif or Sp1-binding sites were detected. A repeated GA-rich region of unknown function was found from position −218 to −410. The Nas1 promoter contains a number of potential cis-acting elements recognized by well characterized transcription factors that may play a role in the basal or chronic regulation of the Nas1 gene. These include two AP-1 sites, one AP-2 site, one AP-4 site, two CAAT/enhancer-binding protein (C/EBP) binding sites, three Oct-1 sites, three NF-Y sites, and two NFAT...
The programs used for predicting the transmembrane spanning domains were TopPred2 (23), TMPred (55), and Sosui (56). There were many GATA-1-binding sites, located at nucleotides –153, –400, –605, –682, –928, –948, –1222, –1348, –2088, and –3030. Consensus sequences for the binding of other transcription factors activated by mitogenic or differentiation signals (c-Elk-1, Sox-5, hepatic nuclear factor 4, c-Fos, c-Jun, c-Myb, and CREB) were found (28). Within the same region, the sequence 5′-AGCTCA-3′ bears strong resemblance to the consensus inverted repeat sequence (5′-AGGTCA—TGACCT-3′) of TREs. This same sequence also had similarities to the consensus core binding motif (5′-AGCTCA-3′) of VDREs (29). Region B (525 to 550) contains a consensus DR6-type structure (5′-GTGTGAA-3′) with similarities to mouse osteopontin VDRE (28). Within the same region, the sequence 5′-AGCTCA-3′ bears strong resemblance to the consensus inverted repeat sequence (5′-AGGTCA—TGACCT-3′) of TREs. This same sequence also had similarities to the consensus core binding motif (5′-AGCTCA-3′) of VDREs (29).

**TABLE II**

| Intron number | Exon size (bp) | Codon phase | Exon number | Exon size (bp) |
|---------------|----------------|-------------|-------------|----------------|
| 1             | 15,000         | 0           | 1          | 127            |
| 2             | 12,900         | 0           | 2          | 129            |
| 3             | 2,500          | II          | 3          | 137            |
| 4             | 600            | I           | 4          | 188            |
| 5             | 70             | II          | 5          | 58             |
| 6             | 13,300         | I           | 6          | 49             |
| 7             | 10,000         | II          | 7          | 134            |
| 8             | 3,500          | II          | 8          | 138            |
| 9             | 560            | II          | 9          | 96             |
| 10            | 3,100          | II          | 10         | 102            |
| 11            | 2,400          | I           | 11         | 107            |
| 12            | 4,800          | I           | 12         | 110            |
| 13            | 1,250          | I           | 13         | 162            |
| 14            | 1,500          | Met         | 14         | 138            |

sites. There were many GATA-1-binding sites, located at nucleotides –153, –400, –605, –682, –928, –948, –1222, –1348, –2088, and –3030. Consensus sequences for the binding of other transcription factors activated by mitogenic or differentiation signals (c-Elk-1, Sox-5, hepatic nuclear factor 4, upstream stimulating factor, FREAC4, and Pit-1) are also present (Fig. 7). However, the evaluation of many of these sites in relation to known Nas1 functions will require additional studies.

**Structure of Putative Steroid-Thyroid Hormone-responsive Elements in the Nas1 Promoter**—Three regions, named A, B and C, containing direct repeat-like sequences similar to steroid-thyroid hormone-responsive elements were found (Fig. 7). Within the region A (–2549 to –2515), the sequence 5′-AGTACGaaG/TGCCT-3′ bears strong resemblance to the consensus inverted repeat sequence (5′-AGGTCA—TGACCT-3′) of TREs.
Fig. 5. Nas1 transcription products. A, Northern analysis of total kidney mRNA. Top panel, total RNA (25 μg) from mouse kidney, ileum, duodenum/jejunum, colon, and liver was hybridized to a full-length 2.2-kb mNaSi-1 cDNA probe. Lower panel, after stripping, the same membrane was hybridized to a 1.3-kb β-actin cDNA probe, as a control for RNA quality and loading. Exposure times were 24 h for mNaSi-1 probe and 1 h for β-actin probe. B, 3′-RACE. Total RNA was reverse-transcribed with an oligo(dT)/adapter primer and PCR-amplified using an antisense adapter primer and sense mNaSi-1-specific primer as indicated. C, structure of the Nas1 transcription products. Two forms of mNaSi-1 mRNA were derived from alternative polyadenylation (2.2 kb). Exons (2.1 kb) are indicated by filled bars in vitro indicated. An antisense adapter primer and sense mNaSi-1-specific primer was transcribed with an oligo(dT)/adapter primer and PCR-amplified using an antisense adapter primer and sense mNaSi-1-specific primer as indicated. D, mapping the transcription start site of Nas1 using primer extension and 5′-RACE. A, primers RN44 and RN88 (see C) were mixed with either no RNA (lanes 6 and 10) or with 10 μg of total RNA prepared from mouse kidney or liver, as indicated. A control primer extension reaction was also included. The primers were extended with avian myeloblastosis virus-reverse transcriptase at 42 °C for 30 min. The reaction products were size-fractionated on a 6% denaturing polyacrylamide gel, followed by exposure to film for 1.5 (lanes 1 and 2) or 48 h (lanes 3–10). The sizes of the primer extension products were determined by their migration relative to a molecular weight marker (HindIII-digested λx174, labeled with [γ-32P]dATP). B, total RNA (5 μg) isolated from mouse kidney was reverse-transcribed using primer RN535. After two rounds of PCR amplification with primer RN226 (see C), a 277-bp fragment was obtained (lane 2). DNA size markers (lane 1) and a PCR blank (lane 3) are shown. C, 5′-flanking sequence of the Nas1 gene. Primers used in primer extension and 5′-RACE experiments are indicated. The ATG translation initiation site is boxed. The transcription start sites as mapped by primer extension (defined as position +1) and 5′-RACE are indicated by the arrow and asterisk, respectively. Identical results were obtained in two additional experiments.

Discussion

NaSi-1 is a high affinity Na⁺-sulfate cotransporter present on the BBM of the renal proximal tubule (9) and ileum (27). The potential transcriptional activity of the Nas1 promoter in response to 1,25(OH)₂D₃ was initially tested using the pNas1-1203 construct (Fig. 9A). Cotransfection of pNas1-1203 and the VDR expression vector into OK cells did not result in increased luciferase activity, when compared with transfection with pNas1-1203 alone. In contrast, in OK cells expressing both the VDR and hRXRα, the increase in luciferase activity was 8.9-fold higher than with activation of pNas1-1203 alone. Under these conditions, in the presence of 0.5 and 50 nM 1,25(OH)₂D₃, the promoter activity was further increased by 1.8- and 4.1-fold, respectively (Fig. 9A). Similar experiments were performed for the pNas1-3229, pNas1-1203, pNas1-457, and pNas1-1203R constructs, and the effect of 1,25(OH)₂D₃ in OK cells coexpressing the VDR and hRXRα is summarized in Fig. 9B. When transfected with pNas1-3229 or pNas1-1203 constructs, the luciferase activity increased markedly upon exposure of cells to 0.5 or 50 nM 1,25(OH)₂D₃. In contrast, the luciferase activity in OK cells transfected with pNas1-457 or pNas1-1203R was not affected by 1,25(OH)₂D₃ (Fig. 9B).
tissue distribution, hormonal regulation, and characteristics of expressed activity of NaSi-1 suggest that this transporter may play a crucial role in the maintenance of sulfate homeostasis.

Our report describes the first characterization of the genomic structure and fine chromosomal localization of a mammalian Na\(^+\)-coupled sulfate transporter gene. We also demonstrate that:

![FIG. 7. Nucleotide sequence of the 5' region of the Nas1 gene.](image)

- The translation initiation ATG codon is double underlined.
- The TATA and CAAT boxes, as well as putative transcription factor binding motifs, are underlined and labeled A–C, respectively.
- The GA-rich region of unknown function is in italic uppercase.

![FIG. 8. Transcriptional activity of the Nas1 promoter region.](image)

- OR (filled bars), COS-1 (gray bars), and NIH3T3 (open bars) cells were transiently transfected using LipofectAMINE 2000 with 0.8 \(\mu\)g of reporter vector containing the luciferase gene under the control of the Nas1 promoter and 0.8 \(\mu\)g of the pBGS6Gal control plasmid.
- Transfected cells were harvested after 24 h and assayed for luciferase and \(\beta\)-galactosidase activity. Data are shown relative to activity observed with the pGL-3 vector (promoterless vector).
- The pGL-3 control vector (containing the SV40 promoter) was used as a positive control. Data are means \(\pm\) S.D. from triplicate determinations, and the results are representative of three separate experiments.
cell-specific transcriptional activation of Nas1 expression as well as a transactivation of the Nas1 gene expression by 1,25-(OH)2D3.

The characteristics of expressed activity of mNaSi-1 protein in Xenopus oocytes, as well as the overall pattern of mNaSi-1 mRNA tissue distribution, support the finding that we have cloned the mouse proximal tubular BBM Na+-sulfate cotransporter (34). Both kinetic data and tissue distribution were very similar to the rat NaSi-1 (8). Three mNaSi-1 cDNA variants were identified in mouse kidney mRNA. The two main transcripts (2.2 and 2.5 kb) could be detected by Northern analysis, and comparison with the genomic sequence showed that they were derived from alternative polyadenylation, as shown for the two rNaSi-1 transcripts (2.3 and 2.9 kb) detected in rat kidney mRNA (8, 27). The third mNaSi-1 variant (2.1 kb) was considered to be the result of alternative splicing of exon 2. This alternative spliced version of mNaSi-1 could not be detected on Northern blots and did not show significant sulfate transport when injected in Xenopus oocytes, despite the fact that no frameshift was introduced by exon 2 splicing. A possible explanation for the loss of function is that deletion of exon 2, encoding transmembrane segment 2, would lead to an inverted membrane topology of the protein due to the lack of one transmembrane segment. Alternatively, the removal of transmembrane segment 2 could disrupt the native signal anchor sequence and perturb the sequential mechanism of membrane insertion and folding (35). In view of our data, we conclude that the alternatively spliced mRNA, detected by RT-PCR only, represents a rare transcript that is unlikely to have significant biological relevance.

The mNaSi-1 protein secondary structure model of 13 transmembrane segments contrasts with the secondary structure prediction of rNaSi-1 protein, which was initially predicted to contain 8 transmembrane segments (8). The difference is most probably due to the difference in the prediction method, which was previously based on hydropathy analysis. The new prediction was performed using the TopPred2 program (23), featuring a more precise analysis of the hydropathy data and taking into account the inside positive rule (36). Consensus sequences for N-glycosylation were found at positions 140, 174, and 590; however, according to the 13 transmembrane helices model, only Asn590 is suggested to be extracellular and thus possibly glycosylated. This is consistent with the study of Pajor and Sun (37) showing glycosylation of rabbit NaDC-1 to only occur on Asn578. However, although the 13 transmembrane domain model is likely to represent a better prediction than the 8 transmembrane domain model, additional work is needed to validate this model.

The Nas1 gene contains 15 exons distributed among 75 kb without obvious pattern of exon organization. Although exons encoding transmembrane domains were similar in size, no sequence identity was detected at the nucleotide or amino acid level between them (data not shown), suggesting that these exons did not arise through duplication events. The recently identified SUT-1 transporter shares the highest sequence identity with mNaSi-1; however, its genomic structure is presently unknown, and thus no comparison was possible. The genomic structure of human NaDC-1 gene was recently reported and appeared to be significantly different from the Nas1 gene, containing 12 exons distributed over 23.8 kb of genomic DNA (38). Similarly, the genomic structure of sulfate/anion exchangers differ considerably from the Nas1 gene. The human CLD gene comprises of 21 exons spanning 39 kb (39), whereas the rat Dtdst gene contains only 5 exons spanning approximately...
20 kb (40). In contrast, despite sharing no homology with mNaSi-1 cDNA, the human Na\(^+\)-glucose SGLT1 transporter shares a comparable gene structure with Nas1 consisting of 15 exons distributed among 72 kb (41). Particularly, the exon sizes and their distribution are very similar to that of Nas1. Moreover, most of the 35 members of the SGLT1 gene family share a common core structure of 13 transmembrane helices (42), as is the case for Nas1. Superimposing the Nas1 exon boundaries on the mNaSi-1 protein secondary structure model shows that splicing frequently occurs near membrane/aqueous transitions, as is also observed with the SGLT1 gene (41) and other genes encoding membrane proteins, such as the murine band 3 (43), human skeletal muscle sodium channel (44), and GLUT1-, GLUT2-, and GLUT4-facilitated glucose transporters (45). It remains to be elucidated whether the similarities between the two Na\(^+\)-cotransporters, SGLT1 and Nas1, are the result of a possible common evolutionary origin.

The Nas1 gene was mapped to mouse chromosome 6, close to marker D6Mit170, which has been assigned a map position of 4.0 centimorgans. The mouse chromosome 6 region from centromere to map position 28 centimorgans is a region of conserved synteny with human chromosome 7. It contains 46 identified genes whose human homologues map to chromosome 7, between regions q14 and q35. Within this region, only one gene (centromere autoantigen E gene) maps to another chromosome (4q24-q25). Altogether these linkage data suggest that the human homologue of the Nas1 gene most likely resides on human chromosome 7q. Interestingly, the human SUT-1 gene, which displays 49% amino acid identity to mNaSi-1, was mapped to 7q33, close to 7q32 (26). Due to their high protein identity and possible chromosomal colocalization, studies in humans are warranted to determine whether the SUT-1 and NAS1 genes could have derived from a gene duplication event. A similar situation was described previously for the DRA and PDS genes, encoding for sulfate and chloride anion exchangers sharing 45% homology and both residing on human chromosome 7q21–31.1 (46).

Transcription initiation of the Nas1 gene occurs at a single site and is under the control of an atypical TATA box located 29 bp upstream of +1, yielding mRNA with a short 26-bp 5'-UTR. The core and flanking residues of the atypical TATA box, TTAT_0TTAAC, differ from the extended canonical sequence (G/C) TAT_0A(A/T)AA(G/A) by having a T in the −3 position, a T in the +1 position, and a C in the +5 position. Nonetheless, a recent study showed that these bases can be present in these positions but at low frequencies (8, 8, and 11%, respectively) (47). Interestingly, the promoter region is AT-rich (61%), rather than GC-rich, a feature that, together with the unique transcription start site, has been observed for genes that are regulated during development and differentiation (48). The AT-rich feature is also consistent with the restricted pattern of Nas1 gene expression since GC-rich promoters are commonly associated with widely expressed "housekeeping" genes. The role of Nas1 during development and differentiation is yet unknown. However, it is interesting to note the presence of multiple binding sites for Sox-5, a transcription factor that has critical roles in the regulation of numerous developmental processes; upstream stimulating factor (USF), a ubiquitous factor involved in development; and hepatic nuclear factor 4 (HNF-4), a thyroid hormone receptor-like factor expressed in kidney from day 10.5 post-coitum and involved in development. In addition, two potential binding sites for FREAC-4, a transcription factor predominantly expressed in kidney (49) and having important roles in embryonic development, as well as regulation of tissue-specific gene expression (50, 51), were found. FREAC-4 has recently been shown to be regulated by the c-Ets-1 proto-oncogene (51). Interestingly, a c-Ets-1 putative binding site was also found in the Nas1 promoter region.

When placed upstream of a reporter gene, the Nas1 promoter could initiate basal gene transcription in a cell-specific manner, since the promoter was only active in OK cells and not in COS-1 or NIH3T3 cells, suggesting tissue specificity of promoter activity. Only FREAC-4-binding sites were identified as potential cis-acting elements associated with kidney-specific gene expression in this region, but their actual role in the cell-specific expression of Nas1 remains unknown.

In a recent study, vitamin D was shown to modulate renal Na\(^+\)-sulfate cotransport (10). Vitamin D-deficient rats showed lower plasma sulfate levels and an increased fractional excretion of sulfate, which correlated with decreases in BBM Na\(^+\)-sulfate cotransport activity and mNaSi-1 protein and mRNA abundance. Moreover, this modulation was shown to be the result of a direct effect of vitamin D, with no independent action of parathyroid hormone or calcium levels (10). The data presented here extend these observations by demonstrating that vitamin D and VDR/RXR\(_\alpha\) transactivated the Nas1 promoter in OK cells. A comparable transactivation by VDR and 1,25-(OH)\(_2\)D\(_3\) was also observed for the renal Na\(^+\)-dependent phosphate transporter gene, NPT2 (52). Our data suggest that the previously reported effect of vitamin D on sulfate homeostasis (10) may, at least in part, be mediated by a transcriptional activation of the Nas1 gene. Although the first 1.2 kb of Nas1 promoter is sufficient for vitamin D transactivation, we cannot rule out that a further upstream region may play a role in this phenomenon, and more detailed studies are required to identify the specific VDRE loci in the Nas1 promoter.

Finally, the presence of five putative GREs and a typical TRE in the Nas1 promoter may also be of significant importance for Nas1 gene regulation and hormonal control of sulfate homeostasis. Glucocorticoids have been shown to regulate renal Na\(^+\)-sulfate cotransport at the BBM level (53), and experimentally induced hypothyroidism in rats led to a decrease of NaSi-1 mRNA and protein levels with no change in membrane fluidity, suggesting a possible down-regulation of the Nas1 gene (54).

In summary, we have isolated and characterized the murine Na\(^+\)-sulfate cotransporter cDNA, gene, and promoter region. In addition, we have investigated its expression in murine tissues, determined its chromosomal localization, identified cDNA variants, and demonstrated that this gene can be transcriptionally activated by 1,25-(OH)\(_2\)D\(_3\) in a renal cell line. This study provides the framework for a more detailed analysis of Nas1 gene expression through the characterization of Nas1 promoter function and the tools required for assessing the role of Nas1 in the maintenance of sulfate homeostasis through the generation and analysis of Nas1-deficient mice.

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