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Expression cloning of rat renal Na^+/SO_4^{2-} cotransport

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ABSTRACT **Injection of rat kidney cortex mRNA into *Xenopus laevis* oocytes leads to a stimulation of Na^+-dependent SO_4^{2-} uptake. Based on this information, we have isolated from a corresponding library a cDNA (NaSi-1) that is most likely related to a Na^+/SO_4^{2-} cotransport system. NaSi-1 cRNA leads in a time- and dose-dependent manner to specific stimulation of Na^+-dependent SO_4^{2-} uptake in oocytes. The apparent affinity constants of the NaSi-1 cRNA-expressed transport resemble those of Na^+/SO_4^{2-} cotransport in brush-border membrane. The NaSi-1 cDNA contains 2239 bp [including a poly(A) tail] and encodes a protein of 595 amino acids (66.05 kDa); the hydropathy profile suggests at least eight membrane-spanning regions. *In vitro* translation of NaSi-1 cRNA results in a protein of the expected size and suggests glycosylation. Northern blot analysis shows signals of 2.3 and 2.9 kb in kidney (more abundant in cortex than in papilla/medulla) and in mucosa of small intestine of rats. The above data indicate that we have structurally identified a membrane protein involved in renal and small-intestinal brush-border membrane Na^+/SO_4^{2-} cotransport.**

The mammalian kidney plays an important role in the maintenance of SO_4^{2-} homeostasis. Urinary excretion of SO_4^{2-} is ≈10% of the filtered load; the tubular reabsorption is mainly achieved by a Na^+-dependent (secondary-active) transport mechanism located in the proximal tubules. Studies with brush-border membrane vesicles isolated from kidney cortex of a variety of animal species have identified a Na^+/SO_4^{2-} cotransport system, with an apparent K_m for Na^+ between 25 and 50 mM and a Hill coefficient exceeding unity; the apparent K_m for SO_4^{2-} is between 0.5 and 1 mM. Na^+/SO_4^{2-} cotransport interacts with other oxyanions such as thiosulfate, but not with phosphate (for review see ref. 1; see also refs. 2–8). Small-intestinal brush-border membranes also contain a Na^+/SO_4^{2-} cotransport system; this transport system has properties similar to those of the renal and has its highest activity in ileal brush-border membranes (e.g., ref. 3). In the proximal tubule, transcellular transport (reabsorption) is completed at the basolateral cell surface, most likely by an anion-exchange mechanism shared by bicarbonate and hydroxyl ions as well as by a variety of organic anions (1, 2).

Na^+/SO_4^{2-} cotransport might be an ideal target mechanism for physiological regulation of renal SO_4^{2-} reabsorption, similar to brush-border membrane Na^+/P_i cotransport (1, 9). Glucocorticoid treatment led to a reduction in chicken renal brush-border Na^+/SO_4^{2-} cotransport activity (8), whereas treatment with thyroid hormone produced an increased Na^+/SO_4^{2-} cotransport activity in mouse renal brush-border membranes (10). Dietary sulfate supply also seems to modulate renal brush-border membrane Na^+/SO_4^{2-} cotransport activity (11, 12).

Until now, only a few of the renal brush-border membrane Na^+/solute cotransport systems have been structurally identified [e.g., Na^+/D-glucose cotransport (13, 14) Na^+/P_i cotransport (15, 16)]. In addition to obtaining structure–function information, a molecular identification of such transport systems is a mandatory prerequisite for future studies on the cellular mechanisms involved in regulatory control of proximal-tubular solute transport. Here we describe the expression cloning of rat renal cortex Na^+/SO_4^{2-} cotransport, using *Xenopus laevis* oocytes. The identified cDNA (NaSi-1) encodes a protein of ≈66 kDa with at least eight putative transmembrane regions.† Kinetic properties of the expressed uptake, as well as tissue distribution of NaSi-1-related mRNAs, strongly suggest that NaSi-1 is closely related to Na^+/SO_4^{2-} cotransport activity of brush-border membranes in renal proximal tubules and small intestine.

EXPERIMENTAL PROCEDURES

***Xenopus laevis* Oocytes and Transport Assay.** Methods for handling of oocytes and the assay for transport have been described (15–18). Oocytes were injected with 50 nl of water without cRNA or with cRNA at 0.005–1 μg/μl. Occasionally we have also injected total and/or size-fractionated rat kidney cortex poly(A)$^+$ RNA (maximally 20 ng per oocyte). After 1–6 days, uptake of $^{35}SO_4^{2-}$, of methyl α-D-[^{14}C]glucopyranoside, of L-[^{3}H]leucine, of $^{32}P_i$, and of L-[^{3}H]arginine (New England Nuclear Radiochemicals) was measured in either the presence or absence of Na^+, as described (15–20).

Isolation of RNA and mRNA. RNA was extracted (18, 19) from various tissues and, when specified, poly(A)$^+$ RNA was isolated and size-fractionated by sucrose density gradient centrifugation (16, 18, 21).

Construction and Screening of a cDNA Library. A directional cDNA library was constructed by using size-selected rat kidney cortex poly(A)$^+$ RNA that had been shown to maximally induce expression of Na^+-dependent SO_4^{2-} transport activity in oocytes (2–3 kb; data not shown). The cDNA library was constructed by using a commercial kit (SuperScript plasmid system, pSPORT1 vector; GIBCO/BRL) following precisely as instructed by the supplier and contained about 2×10^5 colonies, of which 40,000 have been screened by a sib-selection procedure; initial pools for screening contained about 1000 colonies. Plasmid DNA was isolated by standard procedures (alkaline lysis and using Qiagen columns; Kontron, Zürich). Plasmids were linearized with *Not* I and used for *in vitro* synthesis of cRNA, including capping, with T7 RNA polymerase (Promega) (22). Synthesized cRNA was dissolved in water for further use.

DNA Sequencing/Primer Extension. Sequencing was carried out by the chain-termination method using a T7 polymerase sequencing kit (Pharmacia). Both strands of the cDNA insert have been sequenced. Synthetic oligonucleotides were used as primers to proceed through the entire sequence. To determine approximately the length of the 5′ end of the NaSi-1 mRNA transcript, we used the Moloney murine leukemia virus reverse transcriptase RNase H$^-$ primer extension system (Promega), following precisely the

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†The sequence reported in this paper has been deposited in the GenBank database (accession no. L19102).

supplier's protocol, with a 24-mer antisense oligonucleotide (5'-CGG-ATG-ATG-AGA-GGG-AGT-GGC-AAT-3') starting from position +92 on the NaSi-1 sequence. The oligonucleotide was labeled with [γ-^{32}P]ATP and the extended products were analyzed by electrophoresis in a denaturing polyacrylamide gel (as specified by the Promega protocol for the primer extension system).

Northern Analysis. Total RNAs (≈30 μg per lane) were denatured, electrophoresed in 1% agarose/formaldehyde gels and transferred to GeneScreen membranes (DuPont/NEN). cDNA probes of NaSi-1 (full length) and of mouse β-actin (1150-bp *Pst* I fragment; ref. 23) were labeled by random priming (Pharmacia) using [α-^{32}P]dCTP (Amersham). Blots were prehybridized and hybridized in a buffer containing 5× SSPE (1× SSPE is 180 mM NaCl/10 mM NaP_i, pH 7.4/1 mM EDTA), 1% SDS, 5× Denhardt's solution, herring sperm DNA (0.2 mg/ml), and 40% formamide, at 42°C overnight. Blots were washed four times in 2× standard saline citrate (SSC)/0.1% SDS at room temperature, then 20 min in 1× SSC/0.1% SDS at 50°C, followed by 20 min in 0.1 × SSC/0.1% SDS at 60°C (high stringency), with the last step repeated if too much background radiation was present.

***In Vitro* Translation.** NaSi-1 cRNA was translated with a rabbit reticulocyte lysate system in the absence or presence of canine pancreatic microsomes (Promega); we have followed the supplier's protocols with small modifications, as described (24).

RESULTS AND DISCUSSION

Similar to our previous studies on rabbit kidney cortex mRNA (17), injection of poly(A)$^+$ RNA isolated from rat kidney cortex led to an expression of Na^+-dependent SO_4^{2-} uptake in oocytes: 3- to 4-fold over intrinsic activity with total mRNA and 7- to 10-fold with size-fractionated mRNA (fractions containing 2–3 kb mRNA; injection of 15–20 ng, expression measured 3–4 days after injection; data not shown).

We constructed and screened a rat kidney cortex cDNA plasmid library by a sib-selection procedure on the basis of expression of Na^+-dependent SO_4^{2-} transport in *X. laevis* oocytes. As shown in Fig. 1*A*, we obtained a single cDNA-clone (NaSi-1) which specifically stimulated Na^+-dependent SO_4^{2-} uptake. Injection of 1 ng of NaSi-1 cRNA led to >40-fold stimulation of SO_4^{2-} uptake (compared with intrinsic uptake in water-injected oocytes); no apparent stimulation of transport of methyl α-D-glucopyranoside, L-arginine, or L-leucine was observed (Fig. 1*A*). A weak stimulation of Na^+/P_i cotransport was observed, which was subsequently shown to be insignificant (see below and Fig. 1*B*). The increase in SO_4^{2-} transport after injection of NaSi-1-cRNA was entirely Na^+-dependent (Fig. 1*A*). Initial characterization of NaSi-1 cRNA-induced Na^+-dependent SO_4^{2-} uptake documented that the magnitude of expression was related to the amount of cRNA injected (linear between 0.1 and 2.5 ng of cRNA per oocyte, with maximal stimulation between 5 and 20 ng of cRNA per oocyte; 1 day of expression; data not shown) and to the time of expression (up to 6 days, 1 ng of cRNA per oocyte injected; data not shown). Furthermore, under standard conditions (1–2 ng of cRNA injected; up to 4 days of expression) throughout the present study, Na^+-dependent SO_4^{2-} transport was linear in the time of uptake (up to 60 min; data not shown).

Renal brush-border membrane Na^+/SO_4^{2-} cotransport is a unique transport system which is distinct from Na^+/P_i cotransport (see Introduction): Na^+/SO_4^{2-} cotransport is not inhibited by P_i but is shared by thiosulfate. On the other hand, Na^+/P_i cotransport is not inhibited by SO_4^{2-} (for review, see ref. 9); a cDNA encoding rat renal cortex Na^+/P_i cotransport (NaPi-2) has been recently cloned (16). To further determine the selectivity of the presently cloned cotransport system (NaSi-1) and to distinguish it from the Na^+/P_i cotransporter (NaPi-2), we injected oocytes with either NaSi-1 or NaPi-2 cRNA and studied SO_4^{2-} uptake or P_i uptake, respectively, and their mutual inhibition by either P_i, SO_4^{2-}, or thiosulfate. NaSi-1 cRNA-induced SO_4^{2-} uptake was not inhibited by increasing P_i concentrations; as expected, it was inhibited by increasing concentrations of thiosulfate (Fig. 1*B*). NaPi-2 cRNA-induced P_i uptake was inhibited by P_i but not by SO_4^{2-} (Fig. 1*C*). Therefore, the data presented in Fig. 1 document that NaSi-1 cRNA stimulates with high potency a highly selective Na^+/SO_4^{2-} cotransporter; i.e., at physiological concentrations of P_i and SO_4^{2-}, this transporter will interact only

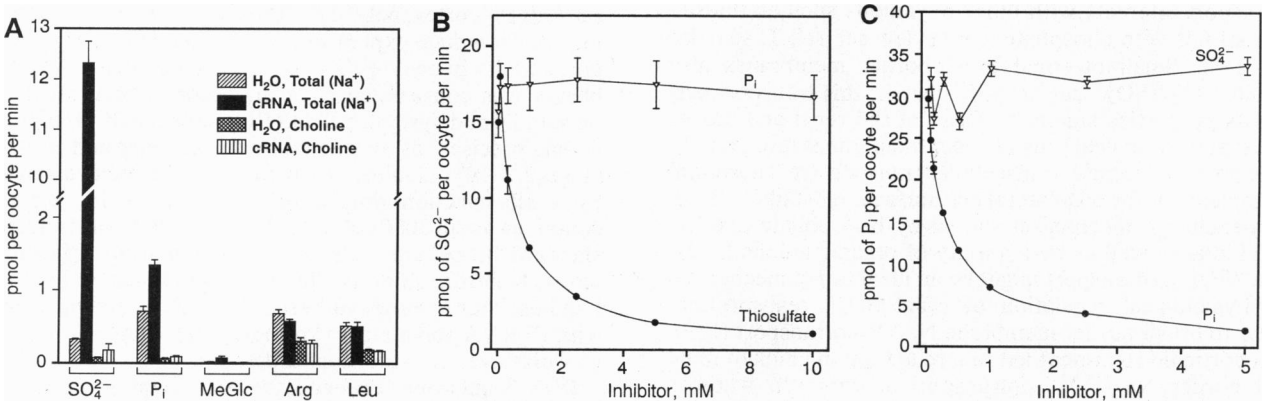

FIG. 1. Cloning of NaSi-1 cDNA and specificity of expressed uptake. (*A*) Oocytes were injected with 50 nl of water or 50 nl of NaSi-1 cRNA (1 ng per oocyte). One day after injection, transport was measured in the presence of Na^+ (100 mM NaCl) and in its absence (100 mM choline chloride) with 0.5 mM $K_2{}^{35}SO_4$ (20–40 μCi/ml; 1 μCi = 37 kBq); 0.5 mM $K_2H^{32}PO_4$ (20–40 μCi/ml); 0.1 mM methyl α-D-[^{3}H]glucopyranoside (12.5 μCi/ml); 50 μM L-[^{3}H]arginine (5 μCi/ml), and 50 μM L-[^{3}H]leucine (5 μCi/ml). Data are shown as means ± SE for 7–10 oocytes per condition and are representative of at least two similar experiments. (*B*) Oocytes were injected with 50 nl of water or 50 nl of NaSi-1 cRNA (1 ng per oocyte). Two days after injection, transport was measured in a Na^+ medium (100 mM NaCl) with 0.5 mM $K_2{}^{35}SO_4$ as substrate (20–40 μCi/ml) and various concentrations of thiosulfate ($K_2S_2O_3$, 0–10 mM) and P_i (K_2HPO_4, 0–10 mM) as inhibitors. Data are shown as means ± SE for 7–10 oocytes per condition and are representative of at least two similar experiments. For thiosulfate, the inhibition constant (K_i) was calculated to be 0.33 mM. (*C*) Oocytes were injected with 50 nl of water or 50 nl of NaPi-2 cRNA (1 ng per oocyte; ref. 16). Two days after injection, transport was measured in a Na^+ medium (100 mM NaCl) with 0.5 mM K_2HPO_4 as substrate (20–40 μCi/ml), and various concentrations of SO_4^{2-} (K_2SO_4, 0–5 mM) and P_i (K_2HPO_4, 0–5 mM) as inhibitors. Data are shown as means ± SE for 7–10 oocytes per condition and are representative of at least two similar experiments. In Fig. 1, SE values not included were smaller than the symbols.

with SO_4^{2-}. However, as indicated by the slight stimulation of P_i transport in NaSi-1-injected oocytes (Fig. 1*A*), this transporter could also show a "weak" interaction with P_i when it is the only anion present.

To further characterize NaSi-1 cRNA-induced SO_4^{2-} uptake, we studied its Na^+ as well as SO_4^{2-} dependence (Fig. 2). SO_4^{2-} dependence showed simple Michaelis–Menten kinetics (K_m = 0.62 ± 0.08 mM; Fig. 2*A*). For the Na^+ interaction of NaSi-1 cRNA-induced transport, a sigmoidal relationship was observed (Fig. 2*B*); these data were fitted to a generalized Hill equation (K_m = 16.8 ± 2.9 mM; Hill coefficient n = 1.8 ± 0.4). The above properties of NaSi-1-induced Na^+-dependent SO_4^{2-} uptake (Fig. 1 *A* and *B*; Fig. 2 *A* and *B*) are in close agreement with the properties of brush-border vesicle Na^+/SO_4^{2-} cotransport (e.g., refs. 3, 5, and 7; for review, see ref. 1).

4,4′-Diisothiocyanatostilbene-2,2′-disulfonate (DIDS) is a known anion-exchange inhibitor that inhibits Na^+-independent SO_4^{2-} transport in rat renal cortex basolateral membranes (4). We observed no effect of DIDS (up to 1 mM) on the NaSi-1-induced Na^+/SO_4^{2-} cotransport activity (data not shown), confirming that NaSi-1 encodes a Na^+-coupled transport system (and not an anion exchanger).

By Northern blot hybridization using full-length NaSi-1-cDNA as a probe, we analyzed tissue/organ distribution in the rat, and species homologies with kidney cortical tissues of various mammalian species. In RNAs from various rat tissues (Fig. 3), two transcripts (2.3 and 2.9 kb) were detected in kidney cortex, kidney medulla/papilla, upper small intestine (duodenum and jejunum) and lower small intestine (ileum). With β-actin as an internal standard, the hybridization signals were stronger in kidney cortex than in kidney medulla/papilla and stronger in lower small intestine (ileum) than in upper small intestine (duodenum and jejunum; Fig. 3). No hybridization signals were observed in RNA from other rat tissues (proximal colon, lung, liver, brain, heart, and skeletal muscle; Fig. 3 and data not shown). The above distribution of NaSi-1 related mRNA(s) is in agreement with

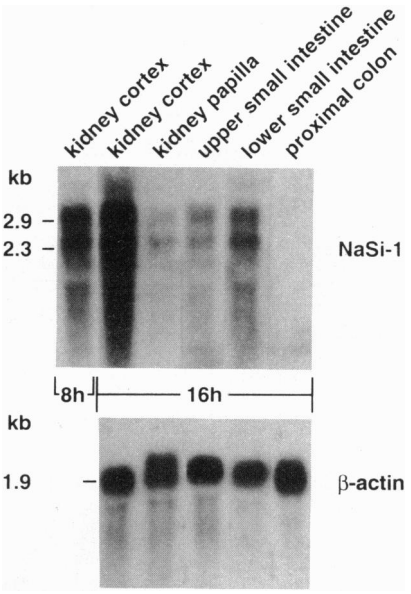

FIG. 3. Northern blot hybridization using full-length NaSi-1 and partial length β-actin cDNA probes. Total RNA samples from various rat tissues were analyzed with an NaSi-1 cDNA probe. Hybridization signals (2.3 kb and 2.9 kb) were observed only with kidney cortex, kidney medulla/papilla, upper small intestine (duodenum and jejunum) and lower small intestine (ileum). The β-actin signals obtained from the same blots are given to provide information on the quality and amount of samples loaded. For kidney cortex, two different exposure times (8 and 16 hr) are given for visualization of the two bands.

NaSi-1 being related to brush-border membrane Na^+/SO_4^{2-} cotransport. Cross-species hybridization of NaSi-1 was observed with kidney cortex RNAs from mouse (2.3 and 2.6 kb), rabbit (2.6 and 3.3 kb), and pig (3.2 kb) (data not shown),

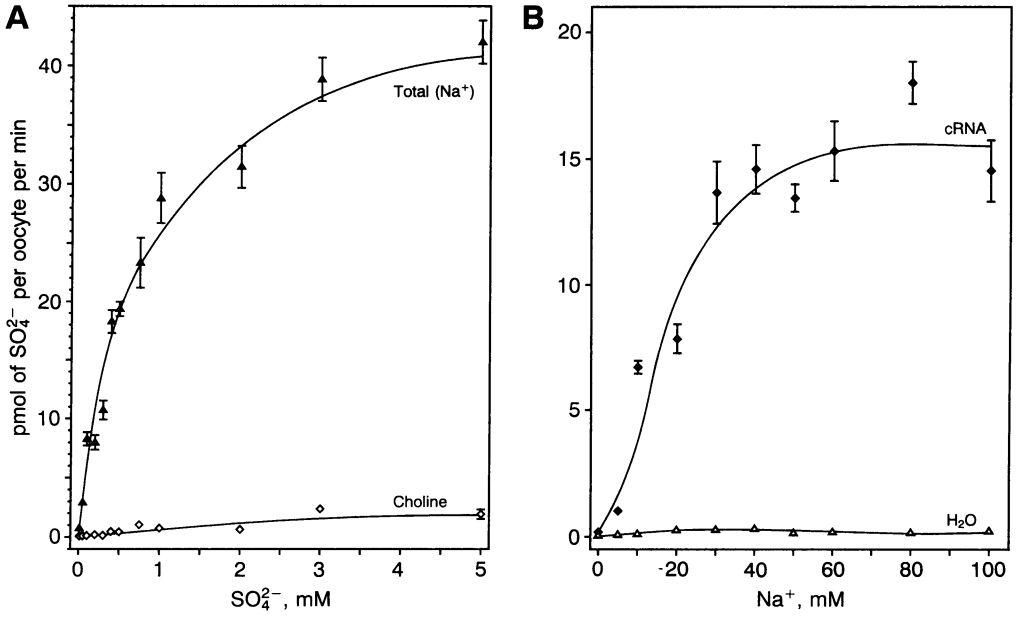

FIG. 2. Na^+ and SO_4^{2-} concentration dependence of NaSi-1 cRNA-induced SO_4^{2-} uptake. (*A*) Oocytes were injected with 50 nl water or 50 nl NaSi-1 cRNA (1 ng per oocyte). Two days later, transport was measured in the presence of Na^+ (100 mM NaCl; ▲) and in its absence (100 mM choline chloride; ◇) with 0.01–5.0 mM K_2SO_4 as substrate (20–40 μCi/ml). Data are shown as means ± SE for 7–10 oocytes per condition, and are representative of four similar experiments. The curve was fitted to a Michaelis–Menten equation by nonlinear regression (K_m = 0.62 ± 0.08 mM; V_{max} = 42.7 ± 2.0 pmol/min). (*B*) Oocytes were injected with 50 nl of water (△) or 50 nl of NaSi-1 cRNA (1 ng per oocyte; ◆). Two days later, transport was measured in the presence of various concentrations of Na^+ (0–100 mM NaCl; isoosmotically replaced by choline chloride) with 0.5 mM K_2SO_4 (20–40 μCi/ml). Data are shown as means ± SE for 7–10 oocytes per condition and are representative of three similar experiments. The curve was fitted to a generalized Hill equation by nonlinear regression (K_m/K_D = 16.8 ± 2.9 mM; apparent V_{max} = 17.0 ± 1.4 pmol/min; n = 1.8 ± 0.4). SE bars not visible were smaller than the symbols.

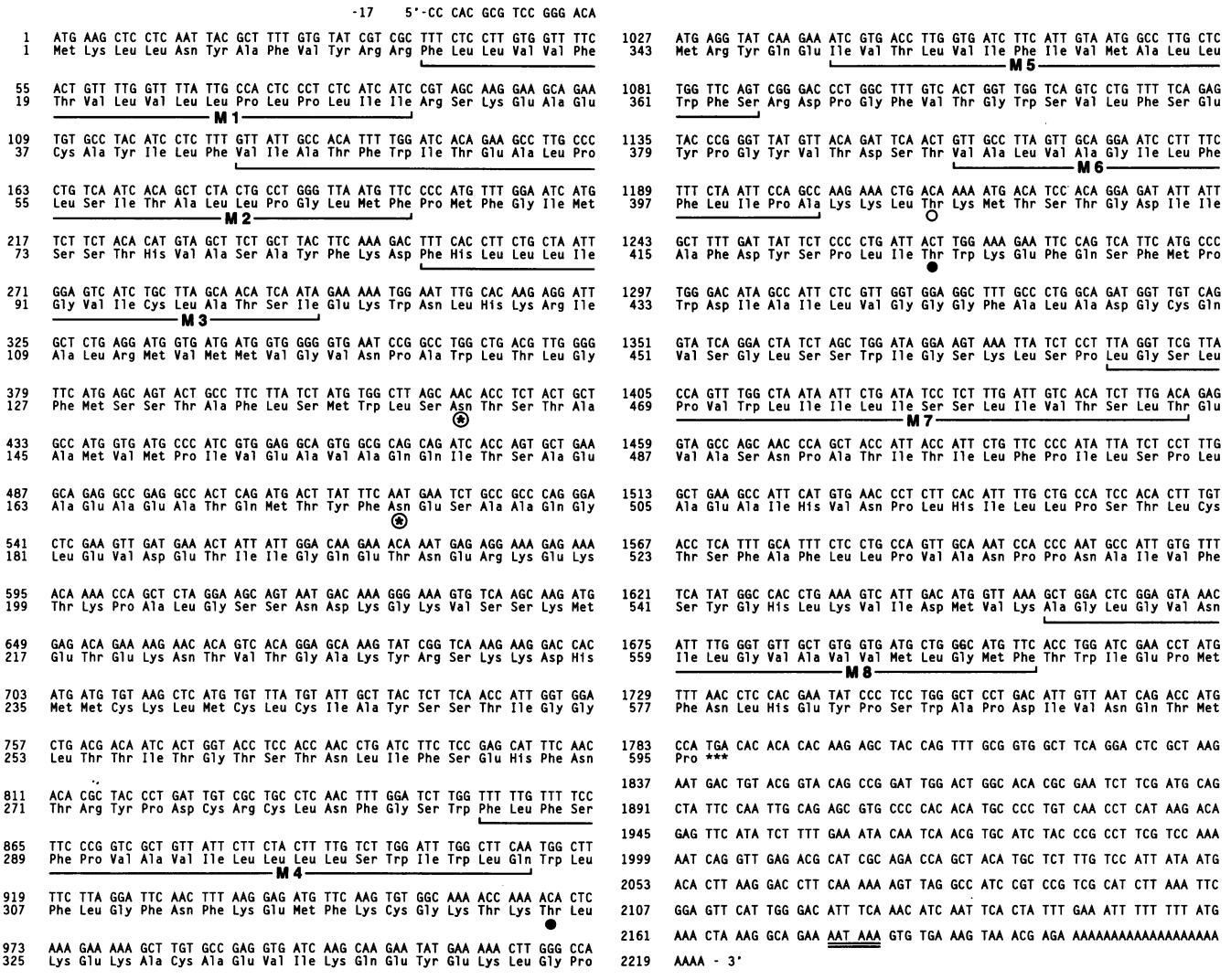

FIG. 4. Nucleotide and predicted amino acid sequence of NaSi-1 cDNA. Nucleotides are numbered beginning with the first ATG initiation codon within a strong Kozak consensus sequence (25); the stop codon (TGA) is indicated by stars. Putative transmembrane domains (M1–M8) are underlined. Potential N-glycosylation sites (aa 140 and 174) are indicated by circled stars; potential phosphorylation sites located at the cytoplasmic surface are labeled (○, protein kinase A; ●, protein kinase C). An additional putative N-glycosylation site is at aa 591 and other potential phosphorylation sites are at aa 213, 218, and 230 for protein kinase C and at position 39 for tyrosine kinase. The polyadenylylation signal preceding the poly(A) tail is doubly underlined.

suggesting that the NaSi-1 transcript may be highly homologous to the corresponding transcripts (Na^+/SO_4^{2-} cotransporters) in other species.

The NaSi-1 cDNA insert was completely sequenced on both strands (Fig. 4). It is 2239 bp long, with 17 nt prior to the first start codon (ATG) and a stop codon (TGA) at position 1786. Thereafter, another 434 nt are present at the 3′ end, with a putative polyadenylylation signal (AATAAA) at position 2176, closely followed by a 24-nt poly(A) tail. The predicted open reading frame is 595 aa long, which corresponds to a protein of 66 kDa (Fig. 4). Due to a potential signal cleavage site at aa 33 (after the first hydrophobic domain; Figs. 4 and 5), the mature protein might differ in size.

To approximately determine whether the cloned NaSi-1 cDNA corresponds to the 2.3-kb mRNA band seen in Northern blots, we performed a primer extension experiment using rat kidney cortex mRNA and an antisense primer starting at nt +92 (see *Experimental Procedures*). The extended product, analyzed in a polyacrylamide gel, was about 110 bp long (data not shown), which is in agreement with the length of the 5′ end of the cloned NaSi-1 cDNA. We therefore believe that the NaSi-1 cDNA corresponds in length to the 2.3-kb mRNA seen in Northern blots (Fig. 3). We also assume that the 2.9-kb mRNA (Fig. 3) is closely related to NaSi-1 and might represent an mRNA with a prolonged 3′ untranslated region

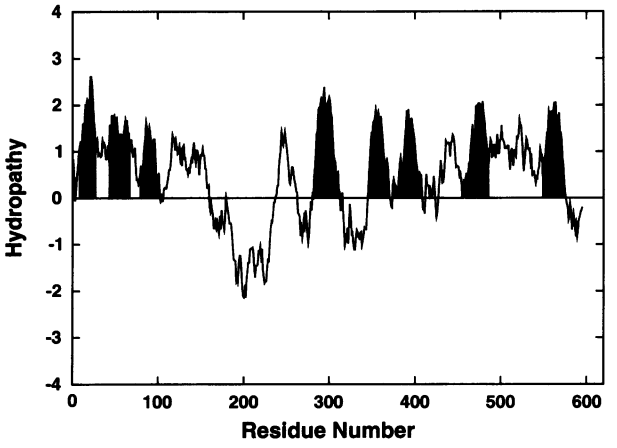

FIG. 5. Hydropathy analysis of the deduced NaSi-1 amino acid sequence by the algorithm of Kyte and Doolittle (26) with a window of 17. Shadowed areas show eight putative transmembrane domains.

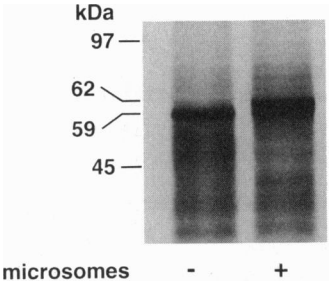

FIG. 6. *In vitro* translation of NaSi-1 cRNA. One microgram of NaSi-1 cRNA was used and the translation products were analyzed directly by SDS/10% PAGE. In the absence of microsomes, the major translation product has an average size of 59 kDa; in the presence of microsomes the major translation product is shifted to 62 kDa, indicating some core glycosylation.

(e.g., due to the use of a different polyadenylylation site; see ref. 24).

In vitro translation of NaSi-1 cRNA with rabbit reticulocyte lysate resulted in a major band of 59 kDa, which was shifted to 62 kDa in the presence of microsomes (Fig. 6), suggesting that under these conditions, core glycosylation at only one of the three putative N-glycosylation sites may occur (Fig. 4). Comparison of the deduced amino acid sequence of NaSi-1 against Swiss-Prot and Protein Identification Resource databases (confirmed on July 23, 1993 by using the BLAST network) revealed no significant overall similarities, especially not to other cloned Na^+/solute cotransport systems. Notably, a conserved region (SOB motif; refs. 14 and 27) found in certain Na^+-dependent mammalian [e.g., SGLT1 family (14, 28) and Na/P_i cotransporters (15, 16)] and nonmammalian cotransport systems (27) is absent in NaSi-1. Hydrophobicity analysis of the deduced amino acid sequence (26, 29) suggests at least eight membrane-spanning domains (Fig. 5) with a cytoplasmic location of both termini and a large putative hydrophilic extracellular loop containing two potential N-glycosylation sites, Asn^{140} and Asn^{174} (Fig. 4). In the suggested secondary model of NaSi-1, three potential phosphorylation sites (Thr^{405} for protein kinase A and Thr^{323} and Thr^{423} for protein kinase C) are present at the cytoplasmic surface (Fig. 4).

Based on the above data, we conclude that we have cloned a rat cDNA closely related to proximal-tubular (and small-intestinal) brush-border membrane Na^+/SO_4^{2-} cotransport; tissue distribution and the characteristics of expressed uptake are in support of this conclusion. The NaSi-1-encoded protein does not show significant homologies to other cloned Na^+/solute cotransport systems. The cloning of a probable brush-border membrane Na^+/SO_4^{2-} cotransporter represents a significant step toward the cellular/molecular understanding of renal/small-intestinal Na^+-dependent solute reabsorption. In addition to providing primary structural information, NaSi-1 may be an invaluable experimental tool for further studies on the physiological regulation of small-intestinal and renal proximal-tubular Na^+/SO_4^{2-} cotransport.

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