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Primary structure and functional expression of a cDNA encoding the thiazide-sensitive, electroneutral sodium–chloride cotransporter

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ABSTRACT **Electroneutral Na^+:Cl^- cotransport systems are involved in a number of important physiological processes including salt absorption and secretion by epithelia and cell volume regulation. One group of Na^+:Cl^- cotransporters is specifically inhibited by the benzothiadiazine (thiazide) class of diuretic agents and can be distinguished from Na^+:K^+:$2Cl^-$ cotransporters based on a lack of K^+ requirement and insensitivity to sulfamoylbenzoic acid diuretics like bumetanide. We report here the isolation of a cDNA encoding a thiazide-sensitive, electroneutral sodium–chloride cotransporter from the winter flounder urinary bladder using an expression cloning strategy. The pharmacological and kinetic characteristics of the cloned cotransporter are consistent with the properties of native thiazide-sensitive sodium–chloride cotransporters in teleost urinary bladder and mammalian renal distal tubule epithelia. The nucleotide sequence predicts a protein of 1023 amino acids (112 kDa) with 12 putative membrane-spanning regions, which is not related to other previously cloned sodium or chloride transporters. Northern hybridization shows two different gene products: a 3.7-kb mRNA localized only to the urinary bladder and a 3.0-kb mRNA present in several non-bladder/kidney tissues.**

Two groups of Na^+:Cl^- cotransporters have been identified based on their different sensitivities to inhibitors (diuretics) and requirements for potassium: the benzothiadiazine (or thiazide)-sensitive Na^+:Cl^- cotransporter (1, 2) and the sulfamoylbenzoic (or bumetanide)-sensitive Na^+:K^+:$2Cl^-$ and Na^+:Cl^- symporters (3, 4). The thiazide-sensitive Na^+:Cl^- cotransporter is the dominant mechanism mediating Cl^- absorption in the early distal tubule of the mammalian kidney (1, 5, 6) and in the urinary bladder of the teleost *Pseudopleuronectes americanus* [winter flounder (2)]. The characteristics of NaCl absorption in both of these renal epithelia are quite similar: (*i*) the magnitudes of Na^+ and Cl^- transport are identical, interdependent, and not affected by the luminal K^+ concentration (2, 5, 7, 8); (*ii*) Na^+ and Cl^- transport across the luminal membrane is highly sensitive to thiazide diuretics (1, 2, 6, 9–11) but resistant to bumetanide (2, 10), features that distinguish it from the loop diuretic-sensitive Na^+:K^+:$2Cl^-$ and Na^+:Cl^- cotransporters found in the thick ascending limb (4, 12); (*iii*) Na^+:Cl^- reabsorption is not affected by either amiloride or stilbenes (1, 2, 11), indicating that simultaneous operation of Na^+:H^+ and Cl^-:HCO_3^- antiporters are not involved in this NaCl transport; and (*iv*) inhibition of Na^+:Cl^- cotransport with thiazide diuretics increases the rate of calcium absorption (6, 13).

Despite >30 years of the clinical use of benzothiadiazines [thiazides (14)] in the treatment of hypertension and edematous states, the structure of the Na^+:Cl^- cotransporter, or thiazide receptor, has not been elucidated. Recently, Ellison *et al.* (15) have identified a protein band of 185 kDa obtained from membranes of rabbit renal cortex exposed to [^{3}H]metolazone that may be a component of the thiazide-sensitive Na^+:Cl^- cotransporter.

In this report we describe the sequence, functional and pharmacological characterization, and tissue-specific expression of a cDNA clone (TSC_{fl}) encoding the electroneutral thiazide-sensitive Na^+:Cl^- cotransporter, isolated by a functional expression strategy from the urinary bladder of the euryhaline teleost *P. americanus* (winter flounder).¶ Northern hybridizations indicate that a related gene product is expressed in a number of tissues outside the kidney.

MATERIALS AND METHODS

RNA Isolation and Poly(A)$^+$ RNA Selection and Fractionation. Eighty winter flounders were collected at the Woods Hole Marine Biological Laboratory (Woods Hole, MA) and at the Mount Desert Island Biological Laboratory. Total RNA was extracted from the entire urinary bladder and other tissues using the guanidine/isothiocyanate method (16). Poly(A)$^+$ RNA was then selected with oligo(dT)-cellulose (type 7; Pharmacia) chromatography and fractionated according to size using preparative agarose gel electrophoresis (1.5% agarose gel in Tris/EDTA buffer) as described (17, 18). The fractionated RNA was obtained in 60 microfuge tubes containing poly(A)$^+$ RNA from <1 kb to ≈12 kb. RNA was stored in a 70% ethanol solution at −20°C.

***Xenopus laevis* Oocytes and Sodium Transport Measurements.** The general techniques and methods for the preparation, handling, and injection of oocytes were similar to those described in detail (18). In brief, oocyte clusters were incubated in a Ca^{2+}-free medium [(mM): 82.5 NaCl, 2 KCl, and 5 Hepes/Tris, pH 7.4] for 1 hr, and individual stage IV–V oocytes were separated using an L-shaped glass tube. Separated oocytes were then manually defolliculated after 90 min of incubation in a collagenase-containing [Boehringer Mannheim; type B (2 mg/ml)] medium [ND96 (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 MgCl, and 5 Hepes/Tris, pH 7.4]. Defolliculated oocytes were injected with total poly(A)$^+$ RNA at a concentration of 0.5 μg/μl or fractionated mRNA or complementary RNA (cRNA) at a concentration of 0.25 μg/μl. $^{22}Na^+$ uptake was measured in groups of 20 oocytes 2–4 days after injection using the following protocol: a 30-min incubation in a Na^+-free ND96 medium [(mM): 96 *N-methyl*-D-glucamine chloride, 2 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5 Hepes/Tris, pH 7.4], followed by a 15-min incubation in a Na^+-free,

Abbreviations: cRNA, complementary RNA; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; CAI, carbonic anhydrase inhibitor.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L11615).

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K^+-free medium [(mM): 98 *N-methyl*-D-glucamine chloride, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5 Hepes/Tris, pH 7.4] containing 1 mM ouabain, 0.1 mM amiloride, and 0.1 mM bumetanide, and finally a 60-min uptake period in a K^+-free medium containing the three transport inhibitors and 20 μCi (1 Ci = 37 GBq) of $^{22}Na^+$ per ml (Amersham). Ouabain was added to prevent $^{22}Na^+$ exit via Na^+:K^+-ATPase and amiloride was added to prevent $^{22}Na^+$ uptake via Na^+ channels or Na^+:H^+ antiporters. Removal of K^+ from, and addition of bumetanide to, the uptake medium prevented $^{22}Na^+$ uptake via the Na^+:K^+:$2Cl^-$ cotransporter endogenously expressed in oocytes (19). To determine the Cl^--dependent fraction of $^{22}Na^+$ uptake, paired groups of oocytes were incubated in uptake media with Cl^- [(mM): 20 NaCl, 76 *N-methyl*-D-glucamine chloride, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes/Tris, pH 7.4] and without Cl^- [(mM): 20 sodium gluconate, 76 mM *N-methyl*-D-glucamine gluconate, 6.0 calcium gluconate, 1 magnesium gluconate, 5 Hepes/Tris, pH 7.4]. All experiments were performed at room temperature (21–22°C). Thiazide sensitivity was assessed using a 0.1 mM concentration of each diuretic.

Library Construction and Clone Isolation. A directional cDNA library was constructed in the plasmid vector pSPORT1 (SuperScript; BRL) using the size-fractionated poly(A)$^+$ RNA that gave rise to the peak of Cl^--dependent, $^{22}Na^+$ uptake. cDNA from 2.5 to 4.5 kb was isolated using GeneClean (Bio 101, La Jolla, CA) and ligated into pSPORT1 plasmid, which was then electroporated into ElectroMAX DH10B cells (BRL). cRNA was transcribed *in vitro*, using the T7 RNA polymerase in the presence of Cap analog (m^7GpppG; Pharmacia), from pools of clones and injected into oocytes. A pool that induced Cl^--dependent and metolazone-sensitive $^{22}Na^+$ uptake was progressively subdivided until a single clone with a cDNA insert of 3.7 kb was isolated (TSC_{fl}). Injection of >1 ng of cRNA, transcribed from the TSC_{fl} clone, into oocytes induced progressive cell swelling, resulting in oocyte lysis within 24 hr. Oocyte lysis was due to massive NaCl uptake, as it could be completely prevented by the addition of 0.1 mM of metolazone to the NaCl-containing ND96 incubation medium or by incubation of the injected oocytes in a modified medium without Na^+ (replaced by *N-methyl*-D-glucamine) or without Cl^- (replaced by isethionate). Thus, all oocytes used for functional characterization of TSC_{fl} were incubated in a Cl^--free ND96 medium for 2–3 days prior to performance of $^{22}Na^+$ uptakes.

Nucleotide Sequencing and Sequence Analysis. Restriction fragments of TSC_{fl} cDNA were subcloned into pSPORT1 (BRL) and both strands of the subcloned cDNA inserts were sequenced by the dideoxy chain-termination method (20) using the Sequenase DNA sequencing kit (United States Biochemical). Regions between subcloned fragments were sequenced using 17-mer primers. The analysis of the nucleotide and the deducted amino acid sequence and the searches from the GenBank (Release 70), European Molecular Biology Laboratory (Release 29), and Swiss-Prot Bank (Release 20) were performed using the GENEWORKS 2.0 software (IntelliGenetics).

Northern Analysis and *in Vitro* Translation. Ten micrograms of total RNA from each nonbladder tissue, 2.5 μg of

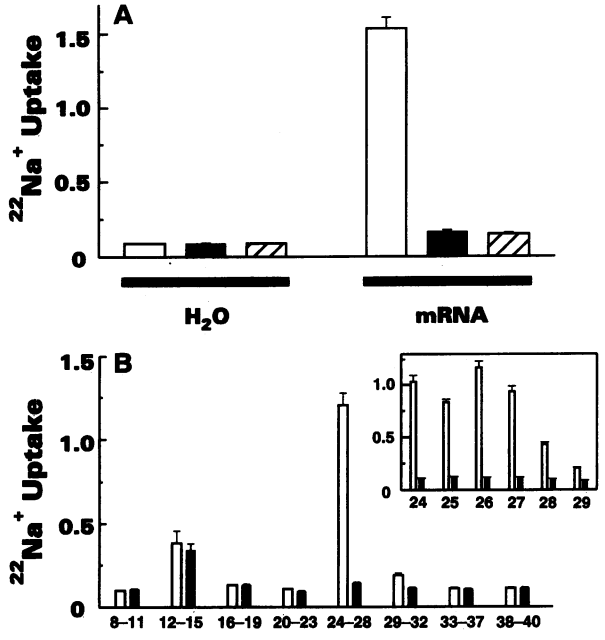

FIG. 1. (*A*) $^{22}Na^+$ uptake in *Xenopus* oocytes injected with water (H_2O) or with 25 ng of flounder bladder poly(A)$^+$ RNA (mRNA) in the presence of Cl^- (□), in the absence of Cl^- (■), and in the presence of Cl^- and 100 μM metolazone (▨). (*B*) $^{22}Na^+$ uptake in oocytes injected with 10-ng pools of size-fractionated poly(A)$^+$ RNA, in the presence (□) or absence (■) of extracellular Cl^-. The mRNA size ranges for pooled fractions 12–15 and 24–28 are 1.3–2.0 kb and 3.4–4.0 kb, respectively. (*Inset*) $^{22}Na^+$ uptake in oocytes injected with fractions 24–29 in the presence (□) or absence (■) of extracellular Cl^-. Each bar represents the mean ± SEM of the uptake values (nmol per oocyte per hr) in 20 oocytes.

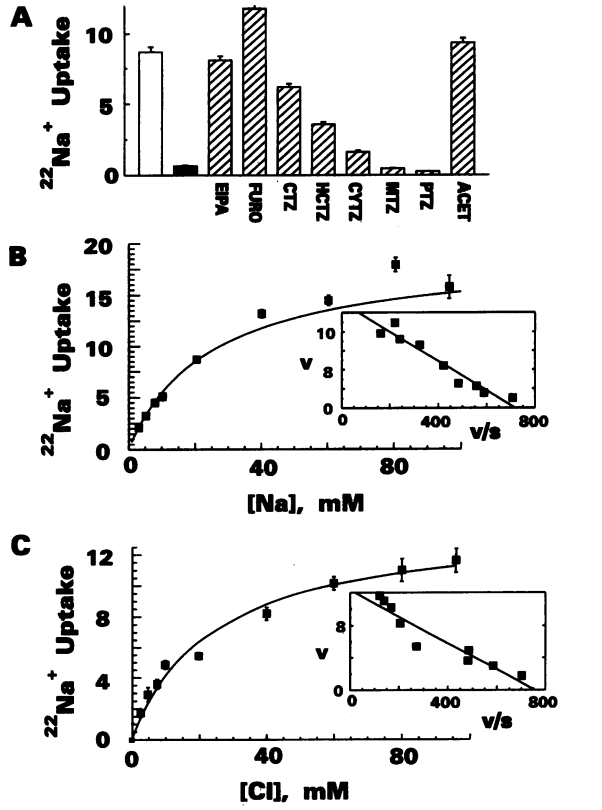

FIG. 2. (*A*) $^{22}Na^+$ uptake in oocytes injected with ≈12 ng of synthetic mRNA (cRNA) transcribed from the TSC_{fl} clone and incubated for 2 days in a modified Cl^--free ND96 medium. Uptake was assessed in the presence (□) or absence (■) of extracellular Cl^- or with one of the following drugs (100 μM): EIPA, chlorothiazide (CTZ), hydrochlorothiazide (HCTZ), cyclothiazide (CYTZ), metolazone (MTZ), polythiazide (PTZ), or acetazolamide (ACET). Furosemide (FURO) was added at a concentration of 1 mM. (*B* and *C*) Na^+ (*B*) and Cl^- (*C*) concentration dependence of $^{22}Na^+$ uptake in oocytes injected with ≈12 ng of TSC_{fl} cRNA. Na^+ and Cl^- were varied from 0 to 96 mM during the uptake. The mean of uptake in H_2O-injected oocytes at the various Na^+ and Cl^- concentrations has been subtracted. (*Insets*) Eadie–Hofstee plots. Each bar represents the mean ± SEM of uptake values (nmol per oocyte per hr) in 20–25 oocytes.

urinary bladder poly(A)$^+$ RNA, and 10 μg of poly(A)$^-$ RNA [i.e., the RNA remaining after isolating poly(A)$^+$ RNA using oligo(dT)-cellulose] were separated by electrophoresis (5% formaldehyde/1.0% agarose gel) and transferred to a nylon membrane (Stratagene). A digoxigenin-UTP-labeled full-length riboprobe (Boehringer Mannheim) was generated from TSC_{fl} by *in vitro* transcription using SP6 RNA polymerase. Hybridizations were performed at 65°C in 5× SSCP/50% formamide/2% SDS/0.1% *N*-lauroylsarcosine/5% blocking reagent (Boehringer Mannheim). The membranes were washed in 0.1× SSCP/0.1% SDS at 65°C and hybridization was detected by chemiluminescence (Boehringer Mannheim). *In vitro* translation of TSC was performed as described (21). In brief, TSC_{fl} cRNA was translated using rabbit reticulocyte lysate (Promega) in the presence or absence of canine pancreatic microsomes (Promega) and endoglycosidase H (Boehringer Mannheim).

Materials. Metolazone was a gift from Fison Pharmaceutical (Rochester, NY), polythiazide was a gift from Pfizer Diagnostics, cyclothiazide was a gift from Eli Liily, and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) was a gift from Merck. Restriction enzymes were from Stratagene and other chemicals were from Sigma, except as noted.

RESULTS AND DISCUSSION

Cloning and Functional Characterization of the Thiazide-Sensitive Na^+:Cl^- Cotransporter. The cloning strategy was based on the ability of mRNA isolated from the urinary bladder of the winter flounder to give rise to thiazide (metolazone)-sensitive, Cl^--dependent $^{22}Na^+$ uptake when injected into *Xenopus* oocytes (Fig. 1*A*). H_2O-injected oocytes exhibited no evidence for endogenous expression of the cotransporter: $^{22}Na^+$ uptake (mean ± SEM) was 0.062 ± 0.001 nmol per oocyte per hr in the presence of Cl^-, 0.064 ± 0.002 nmol per oocyte per hr in the absence of Cl^-, and 0.130 ± 0.003 in the presence of 0.1 mM metolazone. In contrast, the $^{22}Na^+$ uptake observed in oocytes injected with poly(A)$^+$ RNA was about 15-fold higher than in H_2O-injected oocytes [(RNA) 1.432 ± 0.166 vs. (H_2O) 0.062 ± 0.001 nmol per oocyte per hr, $P < 0.01$] and could be nearly completely inhibited either by Cl^- removal (0.143 ± 0.017 nmol per oocyte per hr) or by addition of metolazone to the uptake medium (0.163 ± 0.008

FIG. 3. Nucleotide and predicted amino acid sequences of the cDNA, TSC_{fl}, encoding the thiazide-sensitive Na^+:Cl^- co-transporter. Amino acid residues are numbered on the left; nucleotide positions are numbered on the right. The stop codon (TGA) is indicated by a dot and the poly(A) signal (AATAAA) is double-underlined. The nucleotides surrounding the initiating ATG codon are consistent with the Kozak consensus sequence for eukaryotic mRNA (28). Twelve putative transmembrane segments are underlined (S1–S12). Consensus sites for protein kinases A (#) and C (★) phosphorylation and three potential N-linked glycosylation sites (▲) between S7 and S8 are shown. Two other potential N-linked glycosylation sites at amino acid residues 356 and 543 (not shown) are positioned within the putative membrane segments S6 and S10, respectively.

nmol per oocyte per hr). In addition, the $^{22}Na^+$ uptake observed in mRNA-injected oocytes was not affected by 0.1 mM concentrations of bumetanide, furosemide, acetazolamide, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, amiloride, or EIPA (not shown), and thus, was not due to the expression or activation of amiloride-sensitive Na^+ channels, EIPA-sensitive Na^+:H^+ antiporters (22), or bumetanide-sensitive Na^+:K^+:2Cl^- or Na^+:Cl^- cotransporters (3, 4).

Augmented $^{22}Na^+$ uptake was observed in oocytes injected with two different poly(A)$^+$ RNA size fractions. The uptake induced by fractions 12–15 (1.3–2.0 kb) was 3-fold higher than H_2O [(RNA) 0.440 ± 0.101 vs. (H_2O) 0.107 ± 0.005 nmol per oocyte per hr, $P < 0.01$], but it was not dependent on extracellular Cl^- [(plus Cl^-) 0.440 ± 0.101 vs. (minus Cl^-) 0.322 ± 0.037 nmol per oocyte per hr, P = not significant]. The mechanism of $^{22}Na^+$ uptake in this fraction was not further investigated. In contrast, the tracer Na^+ uptake induced by fractions 25–28 (3.5–4.0 kb) was 12-fold higher than H_2O [(RNA) 1.203 ± 0.071 vs. (H_2O) 0.107 ± 0.005 nmol per oocyte per hr, $P < 0.01$] and was entirely Cl^- dependent [(plus Cl^-) 1.203 ± 0.071 vs. (minus Cl^-) 0.140 ± 0.005 nmol per oocyte per hr, $P < 0.01$]. The peak activity for Cl^--dependent $^{22}Na^+$ uptake was localized to fractions 24–27 (Fig. 1*B Inset*) and these fractions were pooled and used to construct a directional cDNA library. To screen the cDNA library, we prepared sense cRNA by *in vitro* transcription from pools of ≈700 clones, injected the cRNA into oocytes, and assayed for expression of Cl^--dependent $^{22}Na^+$ uptake. One positive pool was progressively subdivided until a single clone with a cDNA insert of 3.7 kb was isolated (TSC_{fl}). Injection of cRNA transcribed from TSC_{fl} into oocytes induced $^{22}Na^+$ uptake that was ≈100-fold higher than in H_2O-injected, control oocytes at 2 days of incubation (Fig. 2*A*) and ≈250-fold higher after 4 days (not shown). Pools of clones that did not contain TSC_{fl} did not give rise to $^{22}Na^+$ uptakes different than controls (not shown). The highly augmented $^{22}Na^+$ uptake seen in TSC_{fl} cRNA-injected oocytes was completely inhibited by removal of extracellular Cl^- but was unaffected by addition of EIPA or furosemide (Fig. 2*A*). In contrast, five different thiazide analogues inhibited the Cl^--dependent $^{22}Na^+$ uptake to varying degrees. The relative inhibitory potency of these thiazides {polythiazide = metolazone [IC_{50} = 3 μM (not shown)] > cyclothiazide > hydrochlorothiazide > chlorothiazide} was similar to that previously shown for inhibition of Cl^--dependent Na^+ absorption in the flounder urinary bladder [assessed as the short circuit current (23)] and for thiazide competition for the high-affinity [^{3}H]metolazone binding site on rat kidney cortical membranes (24).

All thiazides have a sulfamyl group (-SO_2NH_2) that imparts upon them the properties of carbonic anhydrase inhibitors [CAIs (25)], and this CAI activity has been suggested to be important for inhibition of electroneutral NaCl transport in some epithelia (26). Exposure of TSC_{fl} RNA-injected oocytes to 0.1 mM acetazolamide, a potent CAI, however, had no effect on $^{22}Na^+$ uptake (Fig. 2*A*). Moreover, the apparent relative potency of the thiazides to inhibit $^{22}Na^+$ uptake in TSC_{fl}-injected oocytes was different from that expected based on their relative IC_{50} for CAI activity *in vitro* [polythiazide > chlorothiazide > metolazone > hydrochlorothiazide (27)]. Thus the effect of benzothiadiazines to block ion transport by the TSC_{fl} protein is not dependent upon the CAI activity of this class of diuretics.

We also examined the kinetic properties of sodium transport in TSC_{fl} RNA-injected oocytes. The effect of extracellular Na^+ or Cl^- on net $^{22}Na^+$ uptake in TSC_{fl} cRNA-injected oocytes (total uptake − uptake in H_2O-injected controls) is shown in Fig. 2 *B* and *C*, respectively. $^{22}Na^+$ uptake is saturable, consistent with a carrier mechanism. Michaelis constants (K_m) calculated from Eadie–Hofstee plots (*Insets* in Fig. 2 *B* and *C*) were 25.0 ± 0.4 mM and 13.6 ± 0.2 mM for Na^+ and Cl^-, respectively. These K_m values are similar to those reported for the teleost urinary bladder and the mammalian renal distal tubule (2, 5). Hill coefficients for Na^+ and Cl^- were 1.0 ± 0.1 and 0.98 ± 0.1, respectively, consistent with a 1:1 stoichiometry and electroneutral operation of the cotransporter. When taken together, all of these results demonstrate that a single polypeptide is sufficient to express thiazide-sensitive, electroneutral Na^+:Cl^- cotransport activity.

Nucleotide and Amino Acid Sequences. The 3686-bp sequence of the TSC_{fl} cDNA (Fig. 3) contains a single long open reading frame of 3069 nt that encodes a protein of 1023 amino acid residues with a calculated relative molecular mass of 112 kDa. A protein product of the predicted size (≈112 kDa) was generated by translation of the TSC_{fl} transcript using rabbit reticulocyte lysate (not shown). Hydropathy analysis (Fig. 4*A*) revealed the existence of 12 potential membrane-spanning segments and a long hydrophilic COOH terminus of ≈450 amino acid residues. A possible two-dimensional struc-

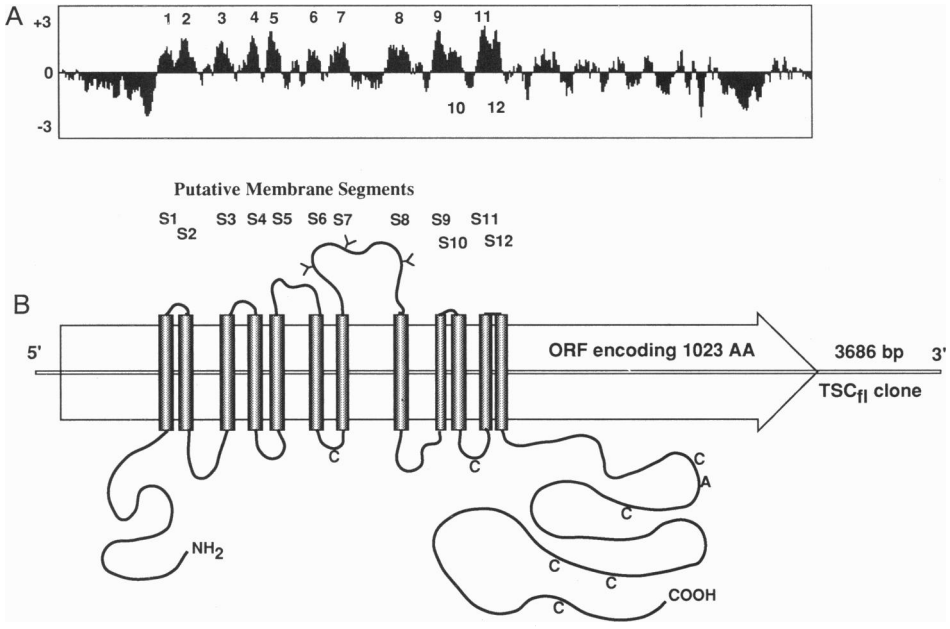

FIG. 4. Hydropathy plot and topology model of the deduced amino acid sequence of TSC_{fl}. (*A*) Hydropathy plot using the Kyte–Doolittle (29) method with a window size of 11 amino acids showing putative transmembrane segments 1–12. (*B*) Membrane topology model of TSC_{fl}. The open horizontal line represents the 3686-bp nucleotide sequence of TSC_{fl} with the open reading frame (ORF) encoding the 1023-amino acid, Na^+:Cl^- cotransporter protein indicated by the large open arrow. The positions of the transmembrane segments (S1–S12), the potential protein kinase A (A) and protein kinase C (C) phosphorylation sites, and the N-linked glycosylation sites (Y) are shown.

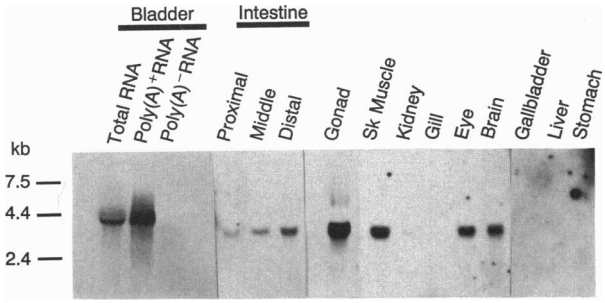

FIG. 5. Tissue distribution of the thiazide-sensitive Na^+:Cl^- cotransporter mRNA. Northern hybridization of a full-length riboprobe of the TSC_{fl} cDNA with RNA isolated from several flounder tissues. The times of autoradiography for the blots were 30 sec for lanes 1–3, 60 min for lanes 4–7 and 12 hr for lanes 8–15. Lanes were loaded with 10 μg of total RNA from each non-bladder tissue, 2.5 μg of urinary bladder poly(A)$^+$ RNA, and 10 μg of urinary bladder poly(A)$^-$ RNA. Sk muscle, skeletal muscle.

tural model is shown in Fig. 4*B* (this model places the NH_2 terminus and COOH terminus in the cytoplasm). Three of the five potential N-linked glycosylation sites were found on the hydrophilic region between the putative transmembrane segments S7 and S8 (Figs. 3 and 4). Two other potential N-linked glycosylation sites (Asn-356 and Asn-543) are within putative membrane-spanning segments S6 and S10, respectively (Fig. 3). Consistent with the cotransporter being glycosylated, translation of TSC_{fl} cRNA in the presence of microsomes yielded a 121-kDa protein band that converted to the 112-kDa band after treatment with endoglycosidase H (not shown). Sequence comparisons using the GenBank, European Molecular Biology Laboratory, and Swiss-Prot data bases did not reveal significant homologies with other known proteins, including other Na^+ or Cl^- transporters. Several consensus sites for protein kinase A and C phosphorylation (30) were found (Fig. 3), although no information is currently available to indicate whether protein kinases alter cotransporter function in native tissues. In addition, the consensus sequence for Na^+-dependent transport systems proposed by Deguchi *et al.* (31) and also identified in the recently cloned mammalian renal Na^+:P_i cotransport system (32) was not found in the deduced TSC_{fl} protein sequence. Thus, to our knowledge, TSC_{fl} is the first member of this unique class of electroneutral Na^+:Cl^- cotransporters to be cloned to date.

Tissue Distribution of TSC_{fl} mRNA. We also examined the tissue distribution of the thiazide-sensitive Na^+:Cl^- cotransporter mRNA by Northern blot analysis performed at high stringency (Fig. 5). Using a full-length riboprobe, two hybridizing bands were observed: an ≈3.7-kb band, corresponding to the TSC_{fl} cDNA, was found exclusively in the urinary bladder and a second smaller band (≈3.0 kb) was found in intestine (distal > middle > proximal), gonad (ovary), skeletal muscle, eye, brain, and kidney. The highest level of expression of the 3-kb gene product was in ovary. The function of the smaller TSC_{fl} gene product is unknown at present. In this regard, however, some investigators have proposed the existence of extrarenal thiazide receptors in hypertensive individuals based on the apparent dissociation of the diuretic action of benzothiadiazines from its long-term antihypertensive effect (33, 34). Recently, the thiazides have also been shown to affect ion transport or tissue function in the rat heart (35), human subcutaneous fat resistance arteries (36), and rabbit gallbladder (37).

Northern blot analysis of rat and mouse renal cortex poly(A)$^+$ RNA performed at low stringency, using riboprobes constructed from TSC_{fl}, revealed three bands of 3.8, 4.5, and 5.5 kb in size (data not shown). Therefore, it should now be possible to clone the mammalian form of this cotransporter and to determine if extrarenal gene expression exists and what role such expression may play in hypertension and cardiovascular function.

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