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Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter

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ABSTRACT **By mediating the coupled movement of Na, K, and Cl ions across the plasma membrane of most animal cells, the bumetanide-sensitive Na-K-Cl cotransporter (NKCC) plays a vital role in the regulation of ionic balance and cell volume. The transporter is a central element in the process of vectorial salt transport in secretory and absorptive epithelia. A cDNA encoding a Na-K-Cl cotransport protein was isolated from a shark rectal gland library by screening with monoclonal antibodies to the native shark cotransporter. The 1191-residue protein predicted from the cDNA sequence has 12 putative transmembrane domains flanked by large cytoplasmic N and C termini. Regulatory phosphoacceptor residues in isolated peptides are identified as Thr-189 and Thr-1114 in the predicted sequence. Northern blot analysis identified a 7.4-kb mRNA in rectal gland and most other shark tissues; a 5.2-kb mRNA was restricted to shark kidney. Homology with an uncharacterized gene from *Caenorhabditis elegans* and with the thiazide-sensitive Na-Cl cotransporter of flounder urinary bladder was found over most of the coding region; shorter stretches of homology were found with a *C. elegans* cDNA and with an uncharacterized gene of cyanobacterium. Human HEK-293 cells have been stably transfected with the shark cDNA and shown to express Na-K-Cl cotransport activity with the bumetanide sensitivity of the shark protein. The expressed transporter is functionally quiescent in the host cells and can be activated by depleting the cells of chloride.**

The Na-K-Cl cotransporter (NKCC) operates in conjunction with the Na pump, a K channel, and a Cl channel to carry out transepithelial salt movement. In secretory epithelia, the importance of this system has been recently underscored by the discovery that mutations in the structure of the Cl-channel protein (CFTR) comprise the defect in cystic fibrosis (1, 2). In an absorptive epithelium, the thick ascending limb of the loop of Henle in the mammalian kidney, the Na-K-Cl cotransporter is the target of the potent "loop diuretic" drugs furosemide and bumetanide (cf. ref. 3).

The rectal gland of the dogfish shark is a model salt-secreting epithelium and is among the richest known sources of the Na-K-Cl cotransport protein (4); it has also been the subject of recent investigations of CFTR, K channels, and Na pumps (5–8). In the secretory cell, cotransporter activity appears to be regulated by the level of intracellular Cl^- through a process involving direct phosphorylation of the transport protein (9, 10).

We have previously prepared monoclonal antibodies to the shark rectal gland cotransporter, a 195-kDa glycosylated protein with a core molecular mass of ≈135 kDa (11). Here we report the cloning of a cDNA encoding the secretory Na-K-Cl cotransporter using these antibodies to screen a shark cDNA expression library.§ A full-length cDNA has been used to direct the expression of the shark cotransporter in a mammalian cell line—the expressed protein is found to retain the low affinity for loop diuretics characteristic of the native shark protein. It is also shown that the expressed cotransporter is subject to regulation in response to ionic concentration changes in the foreign cells.

MATERIALS AND METHODS

Isolation of cDNA and Sequence Analysis. A cDNA library was prepared in λZAP II from 20 μg of poly(A)$^+$-enriched shark (*Squalus acanthias*) rectal gland RNA using oligo(dT) and random primers (Stratagene custom library). Filter transfers of the plated library were screened using a mixture of antibodies J3 and J7, which recognize epitopes in the C-terminal half of the transport protein (11). Three overlapping clones (3B, 9C, 24A) were isolated in two rounds of plaque purification. An additional clone (16-2) was obtained by rescreening the library with the 1.4-kb *Eco*RI/*Eco*RI fragment of the 5′ end of clone 24A.

Both strands of 24A were sequenced by manual dideoxy sequencing (Sequenase; United States Biochemical) of double-stranded *Eco*RI restriction fragments and exonuclease III/S1 nuclease deletion fragments. Both strands of full-length 16-2 cDNA were sequenced using automated sequencing with synthetic oligonucleotide primers and fluorescent dideoxy terminators; the first 1.2 kb of 16-2 and 24A were also confirmed by automated sequencing of single-stranded DNA. The sequence of 24A is identical to that of 16-2 (Fig. 1) except that it extends from base −298 to base 3935, and ATTTTTTTT replaces AGTTTTTTTTAAATTACTAA at −240. TFASTA (12) (Genetics Computer Group, Version 7) and TBLASTN (13) (National Center for Biotechnology Information) were used to search the European Molecular Biology Laboratory and GenBank data bases updated to September, 1993.

RNA Analysis. RNA was prepared from frozen shark tissues by homogenization in guanidinium isothiocyanate and poly(A)$^+$-selected using magnetic beads (Promega PolyATtract). Samples were run on a 1% agarose/formaldehyde gel and transferred to a nylon membrane. Blots were hybridized at 42°C in 50% formamide/2× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/5× Denhardt's solution/1% SDS/200 μg of yeast tRNA per ml/100 μg of herring sperm DNA per ml for 24 hr with a ^{32}P-labeled antisense cRNA produced as a run-off transcript from an *Eco*RI/*Pvu* II fragment of 16-2 cDNA (nt 1235–744), and treated with a final 60-min wash at 65°C in 0.1× SSC/0.5% SDS (1× SSC = 0.15 M NaCl/15 mM sodium citrate).

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§The sequence reported in this paper has been deposited in the GenBank database (NKCC1, accession no. U05958).

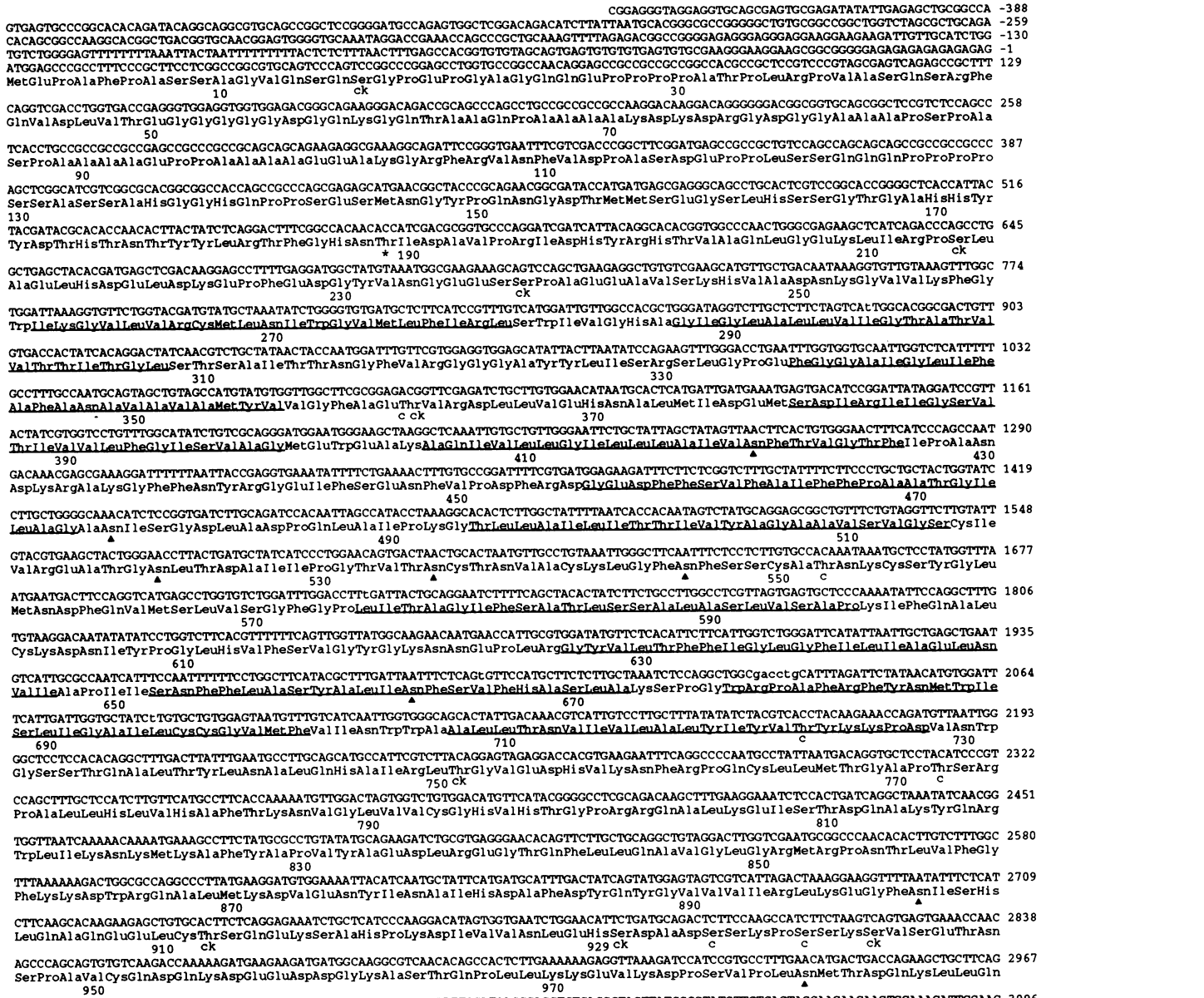

FIG. 1. Nucleotide sequence of cDNA (16-2) encoding the shark rectal gland Na-K-Cl cotransporter (NKCC1) and the inferred amino acid sequence. Amino acid residues are numbered beginning with the initiation methionine. Predicted transmembrane segments are underlined. Potential sites of N-linked glycosylation (▲), consensus targets for protein kinase C (c) and casein kinase II (ck), and biochemically identified phosphorylated residues (*) are labeled.

Transfection of HEK-293 Cells. The 16-2 insert was excised from Bluescript at the *Xba* I and *Eco*RV sites in the polylinker and ligated into the *Xba* I and *Bam*HI sites of the pCB6 vector (14). HEK-293 cells were grown to 60% confluence in Dulbecco's modified Eagle medium containing 10% fetal calf serum and transfected by calcium phosphate coprecipitation with 0.06 μg of 16-2/pCB6 DNA per cm^2. Stably transfected cell lines were selected by growth in 900 μg of G-418 (GIBCO) per ml for 30 days. Approximately 500 colonies were isolated and 202 were tested by dot blot with J3 antibody; 113 were

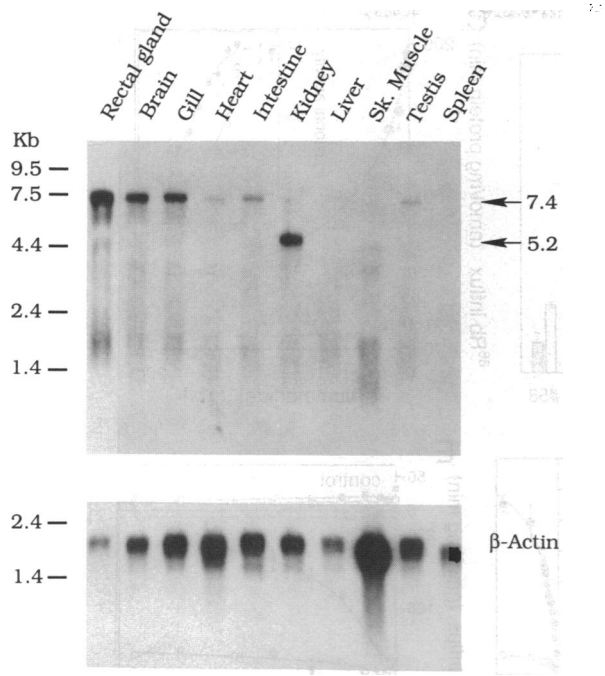

FIG. 2. Northern blot analysis of Na-K-Cl cotransporter expression in shark tissues. mRNA (rectal gland, 1 μg; other tissues, 3–5 μg) was probed with 16-2 cRNA (*Upper*). The exposure time for the rectal gland lane (8 hr, −80°C) was one-fifth that for the rest of the blot. (*Lower*) The blot was reprobed with a β-actin cDNA probe as a control for RNA integrity.

immunoreactive, with a >10-fold range in the amount of expressed protein in different lines. Two cell lines exhibiting robust expression (nos. 8 and 15) were chosen for study. In sectioned pellets of no. 15 cells, immunofluorescence microscopy revealed that shark cotransporter protein was restricted to the plasma membrane (not shown).

Flux Assays. HEK-293 cells were subcultured onto 24-well plates (1:5 split) and grown to confluency (5–8 days) in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Before each flux assay, the cells were incubated in 135 mM sodium gluconate/5 mM potassium gluconate/0.75 mM $CaSO_4$/0.75 mM $MgSO_4$/5 mM glucose/15 mM Hepes, pH 7.4, for 150 min at room temperature, to deplete cells of Cl. The initial rate at which the cells took up ^{86}Rb (0.5 μCi/ml; 1 Ci = 37 GBq) was measured with or without 200 μM bumetanide in 135 mM NaCl/5 mM RbCl/0.75 mM $CaSO_4$/0.75 mM $MgSO_4$/5 mM glucose/0.1 mM ouabain/15 mM Hepes, pH 7.4. Uptake was terminated after 60 sec by addition of, and three rinses in, ice-cold phosphate-buffered saline. In experiments to determine the time course of influx it was found that the approach to isotopic equilibrium is exponential, with $t_{0.5}$ = 2.9 min under these conditions. Cellular extracts (in 500 μl of 1% SDS) were assayed for ^{86}Rb by counting Cerenkov radiation and for protein by the Lowry method.

RESULTS AND DISCUSSION

To isolate cDNAs encoding the Na-K-Cl cotransporter (NKCC), a shark rectal gland cDNA expression library was screened using monoclonal antibodies to the 195-kDa cotransport protein (11). The longest cDNA insert that was obtained (16-2, 5260 bp) includes an open reading frame encoding a protein of 1191 amino acids (Fig. 1). The assigned initiation codon is the first in-frame ATG triplet downstream of a stop codon, and the sequence surrounding it (GAGATGG) conforms to the Kozak consensus for the initiation of eukaryotic translation (15). The 16-2 cDNA lacks a poly(A) tail but includes a polyadenylylation signal (AATAAA) 36 nt upstream from the 3′ terminus. Analysis of the predicted protein sequence by a weight matrix method (16) indicates <5% probability of the presence of a cleavable signal sequence for membrane insertion. The first 1100 bases of the 16-2 cDNA is (G+C)-rich (64%). In addition to the 5′ untranslated region, this region encodes a 145-amino acid N-terminal domain enriched in proline, glutamine, serine, and alanine residues (4.0, 2.2, 2.0, and 2.0 times enriched over average eukaryotic proteins, respectively) and distinguished by numerous amino acid repeats (Pro_4, Gly_5, Gln_3Pro_4, and three examples of Ala_4).

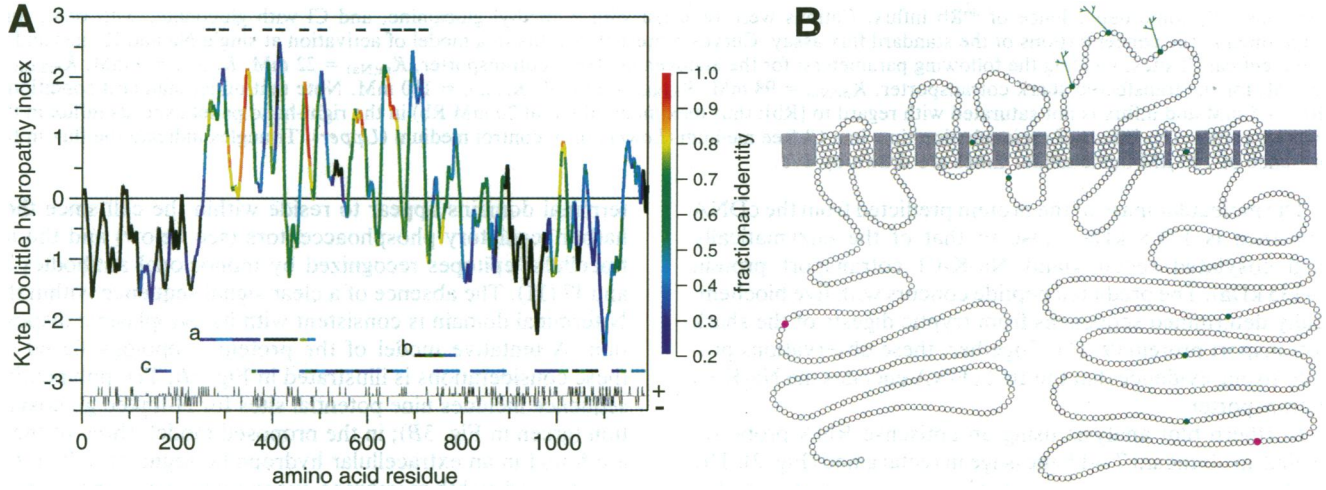

FIG. 3. (*A*) Hydropathy profile of the rectal gland Na-K-Cl cotransporter. The hydropathic index was determined by the Kyte–Doolittle algorithm using a 15-amino acid window. The hydropathy line is color-coded by the fractional identity of the Na-K-Cl cotransporter to the thiazide-sensitive Na-Cl cotransporter averaged over a running 15-amino acid window after aligning the two sequences (there is a 124-residue deletion at the N terminus of the thiazide-sensitive cotransporter and a 45-residue deletion at position 904). Horizontal bars above hydrophobic regions indicate the proposed transmembrane segments. Lines at the bottom of the graph indicate regions of homology with *C. elegans* genomic DNA (a), a *C. elegans* cDNA (b), and a gene from cyanobacterium (c), colored according to the fractional identity to the Na-K-Cl cotransporter. Short lines at the bottom of the figure indicate the location of positive (above the line) and negative (below the line) charged residues. (*B*) Proposed model of the shark Na-K-Cl cotransporter. Circles symbolize the amino acid residues. Potential sites for N-linked glycosylation are highlighted in green; branched lines specify those presumed capable of anchoring oligosaccharide. Identified regulatory phosphoacceptors T-189 and T-1114 are highlighted in red.

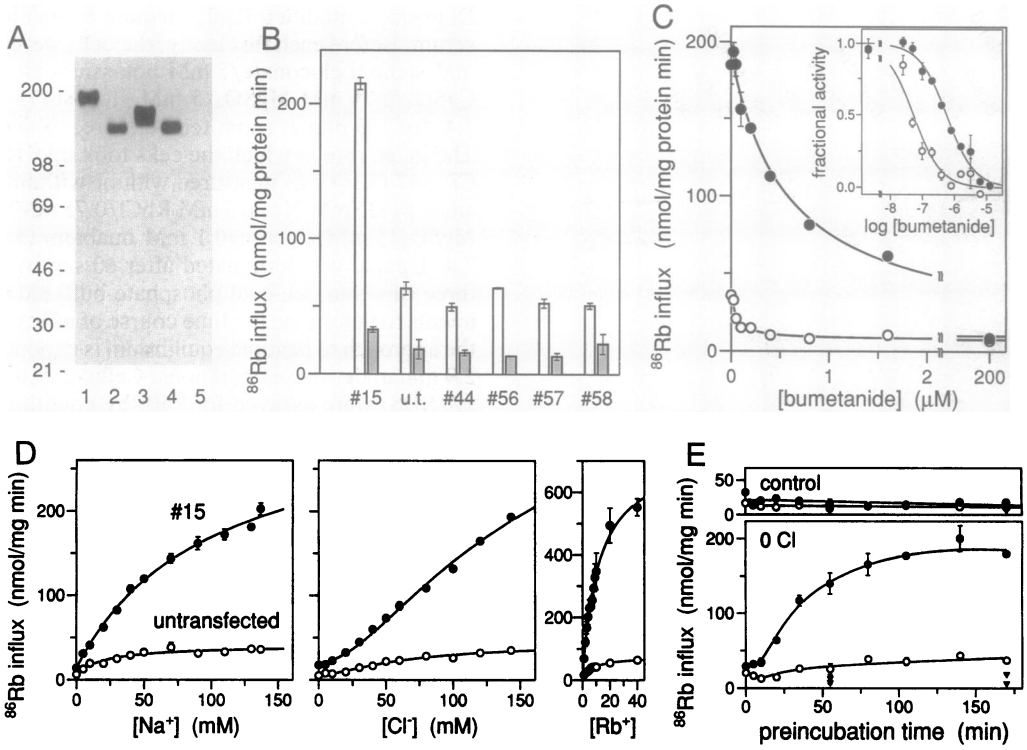

FIG. 4. Characterization of the shark rectal gland Na-K-Cl cotransporter stably expressed in HEK-293 cells. (*A*) Western blot analysis of the native shark cotransporter and of the shark cotransporter expressed in HEK-293 cells. Cellular protein was separated by SDS/PAGE, transferred to nitrocellulose, and probed with monoclonal antibody J3. Membranes are from native rectal gland (lanes 1 and 2), HEK cells stably transfected with 16-2 cDNA (clone no. 15, lanes 3 and 4), and untransfected HEK-293 cells (lane 5). Samples in lanes 2 and 4 were treated with N-glycanase prior to gel electrophoresis. Sizes are indicated in kDa. (*B–E*) ^{86}Rb uptake by wild-type and transfected HEK-293 cells. Confluent cultures of untransfected HEK-293 cells (○) and HEK-293-15 cells (●) were preincubated in medium lacking chloride (gluconate substitution) for 150 min and the activity of the Na-K-Cl cotransporter was determined in a 1-min ^{86}Rb influx assay in flux medium (see *Materials and Methods*). Error bars indicate the range of duplicate samples. (*B*) ^{86}Rb influx in six independent HEK-293 cell lines. Four lines (nos. 44, 56, 57, and 58) were selected that grew in G418 but did not express the shark cotransport protein as determined by J3 immunoblotting. Untransfected HEK-293 cells are designated "u.t.". The initial rate of ^{86}Rb influx was measured in the absence (open bars) and presence (shaded bars) of 200 μM bumetanide. (*C–E*) Untransfected HEK-293 cells (○) and HEK-293-15 cells (●) are compared. (*C*) Differential sensitivity of the endogenous and expressed Na-K-Cl cotransporters to bumetanide. In this experiment, following the exposure to 0 Cl and prior to the flux assay, the cells were exposed to the indicated concentrations of bumetanide for a 30-min period in 20 mM Cl to allow bumetanide binding to take place (cf. ref. 3). The results are fit by a model of bumetanide inhibition at a single site ($K_{0.5}$ = 0.054 and 0.57 μM for untransfected and no. 15 cells, respectively). (*Inset*) The same results as fractional inhibition vs. log [bumetanide], normalized to the maxima and minima of the least squares solutions. (*D*) Ionic dependence of ^{86}Rb influx. Cations were replaced with *N*-methyl glucamine, and Cl with gluconate, with other ions maintained at the concentrations of the standard flux assay. Curves represent best fits to a model of activation at single Na and K sites and at two identical Cl sites, yielding the following parameters: for the endogenous HEK cotransporter, $K_{m(Na)}$ = 22 mM, $K_{m(Rb)}$ = 9 mM, $K_{m2(Cl)}$ = 46 mM; for the transfected shark cotransporter, $K_{m(Na)}$ = 98 mM, $K_{m(Rb)}$ = 12 mM, $K_{m2(Cl)}$ = 110 mM. Note that under standard conditions, [Rb] = 5 mM and influx is not saturated with regard to [Rb]; thus maximum influx (at 20 mM Rb) in the right-hand panel exceeds influx in the left two panels. (*E*) Effect of preincubation time in a Cl-free medium (*Lower*) or in control medium (*Upper*). Triangles indicate the flux in the presence of 200 μM bumetanide. Lines are drawn by eye.

The molecular mass of the protein predicted from the cDNA sequence is 129.8 kDa, close to that of the enzymatically deglycosylated rectal gland Na-K-Cl cotransport protein (≈135 kDa). The predicted peptide concurs with five biochemically determined sequences from tryptic digests of the shark cotransport protein (9–11). Together, these observations provide strong evidence that the 16-2 cDNA encodes the Na-K-Cl cotransporter.

Northern blot analysis using an antisense RNA probe revealed an abundant 7.4-kb message in rectal gland (Fig. 2). The 7.4-kb message is also seen in brain, gill, and intestine and at lower levels in heart, kidney, liver, and testis of the shark (a longer exposure is not shown). Importantly, a second message is observed in shark kidney at 5.2 kb; this may encode a second isoform of the cotransporter involved in absorption of salt across apical membranes of renal tubule cells (17), which we have localized using cotransporter antibodies (18).

Hydropathy analysis (Fig. 3*A*) predicts a central hydrophobic region that could accommodate ≈12 transmembrane helices flanked by large hydrophilic N and C termini. Both terminal domains appear to reside within the cell since they harbor regulatory phosphoacceptors (see below) and the intracellular epitopes recognized by monoclonal antibodies J3 and J7 (11). The absence of a clear signal sequence within the N-terminal domain is consistent with its cytoplasmic disposition. A tentative model of the protein's topology based on these considerations is illustrated in Fig. 3*B*. The polypeptide sequence includes nine potential sites for N-linked glycosylation (green in Fig. 3*B*); in the proposed model, three of these are found in an extracellular hydrophilic segment linking the seventh and eighth membrane-spanning domains and are thus the probable sites of glycan linkage.

The rectal gland cotransporter is known to acquire phosphate at serine and threonine residues when activated by cAMP-dependent (forskolin) and cAMP-independent (cell shrinkage) stimuli; available evidence is consistent with a model in which the response to cAMP is an indirect result of changes in cell [Cl] and involves phosphorylation of the cotransporter by a kinase which is not dependent on cAMP (9, 10). Although no region of the predicted protein conforms

closely to the consensus motif of cAMP-dependent protein kinase, 10 regions do satisfy the requirements for phosphorylation by protein kinase C and at least 10 others for casein kinase II (19) (Fig. 1). We have found that Thr-189 (9) and Thr-1114 (C.L. and B.F., unpublished) are phosphorylated in the rectal gland cell during activation of the cotransporter (9, 10).

When optimally aligned, the Na-K-Cl cotransporter sequence is 47% identical to the recently reported sequence of the thiazide-sensitive Na-Cl cotransporter of flounder urinary bladder (ref. 20; Fig. 3A, color coding). Thus, these proteins appear to represent a new class of membrane transport proteins. Regions that are especially well-conserved are found in the predicted 3rd, 6th, 8th, and 10th transmembrane helices, as well as in the loop connecting helices 2 and 3, and in a short hydrophobic region centered around residue 1013 (Fig. 3A). It seems likely that residues involved in Na and Cl binding and translocation are to be found within these regions.

Database searches also uncovered substantial homology with an unassigned sequence reported by the *C. elegans* genome sequencing project (ref. 21; GenBank accession no. M77697). There is good alignment with the *C. elegans* gene over most of the length of the cotransporter coding region (Fig. 3A, line c), and it seems likely that the *C. elegans* gene also codes for a transport protein. A cDNA from a different *C. elegans* gene was also found to be homologous to the cotransporter sequence (ref. 22; GenBank accession no. M75878; Fig. 3A, line b) as was a gene from the cyanobacterium *Synechococcus* sp. PCC 7002 (ref. 23; GenBank accession no. M18165; Fig. 3A, line a). The latter finding clearly points to an early evolutionary origin of this family of cotransport proteins.

To examine the function of the protein encoded by the 16-2 cDNA, we transfected HEK-293 cells and isolated stably transfected cell lines. Using four monoclonal antibodies that selectively recognize the shark Na-K-Cl cotransporter (J3, J4, J7, and J25), immunoblot analysis revealed that the transfected cells expressed a protein of ≈165 kDa foreign to untransfected HEK cells; this is illustrated for J3 with cell line HEK-293-15 in Fig. 4A, lane 3. The J3 antibody does not recognize the endogenous cotransporter present in untransfected HEK-293 cells (lane 5). Removal of N-linked oligosaccharides with N-glycanase (lane 4) decreased the size of the expressed protein to that of the native shark rectal gland cotransporter after deglycosylation (135 kDa, lane 2), indicating that the entire cotransport peptide is synthesized and partially glycosylated.

The transfected cells exhibited up to 10-fold greater bumetanide-sensitive ^{86}Rb influx than control cells (Fig. 4 *B–E*). As illustrated in Fig. 4*B*, the cotransport activity seen in transfected cells was not associated with the process of G418 selection, since it was not seen in G418-resistant clones that failed to express the shark cotransport protein. Like its counterpart in the rectal gland (4, 24), the expressed shark cotransporter exhibits low sensitivity to loop diuretics; Fig. 4*C* illustrates that it is half-maximally inhibited by 0.57 μM bumetanide. This dose is 10 times that which half-maximally inhibits the endogenous HEK cell cotransporter ($K_{0.5}$ = 0.054 μM; Fig. 4*C*). Thus, the expressed and endogenous cotransporters can be distinguished by their inherently different sensitivity to loop diuretics, consistent with a previously noted difference between secretory and nonsecretory Na-K-Cl cotransporters (4).

As expected of a coupled Na-K-Cl transport process, the expressed ^{86}Rb influx mechanism requires external sodium and chloride and the kinetics of transport are consistent with single binding sites for Na and Rb and with two sites for Cl (Fig. 4*D*). Finally, as illustrated in Fig. 4*E*, expressed Na-K-Cl cotransport activity was only observed when cells were preincubated in a zero chloride medium prior to the ^{86}Rb influx assay (Fig. 4*C*). This is consistent with recent evidence that decreased intracellular Cl is an important stimulus for cotransporter phosphorylation (9, 10) and function (25); in secretory cells, it appears that changes in $[Cl]_i$ may serve as the signal through which the opening of Cl channels in the apical membrane signals an increase in cotransport activity in the basolateral membrane.

These results demonstrate that the protein encoded by the 16-2 cDNA manifests bumetanide-sensitive Na-K-Cl cotransport activity when expressed in HEK-293 cells. The activity of the expressed cotransporter is very similar to that of the native shark rectal gland protein. In addition, it is shown that the activity of the shark cotransporter can be regulated in the foreign cell system and that the necessary Cl-sensing regulatory machinery is present in HEK-293 cells. We have recently used the 16-2 cDNA to obtain homologous cDNAs for the Na-K-Cl cotransporter in human colon (7.5-kb message) (J.A.P., J.-C.X., and B.F., unpublished) and rabbit kidney (ref. 26; NKCC2, 5.1-kb message), thus expanding this emerging family of Cl-dependent transporters.

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