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Alternatively spliced isoforms of the putative renal Na-K-Cl cotransporter are differentially distributed within the rabbit kidney

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ABSTRACT **We have used cDNA probes derived from the secretory form of the Na-K-Cl cotransporter to screen both cortical and medullary rabbit kidney cDNA libraries. A sequence of 4750 bases was identified from multiple clones. The DNA encodes a protein containing 1099 amino acids, which is 61% identical over its length to the secretory Na-K-Cl cotransporter from shark rectal gland. From analysis of amino acid hydropathy, we predict that this putative renal Na-K-Cl cotransporter has 12 transmembrane helices and large N- and C-terminal cytoplasmic regions. Two sites for N-linked glycosylation are predicted on an extracellular loop. Three potential sites for modulation by protein kinase A are in the C-terminal cytoplasmic domain. Most of the isolated renal cDNA clones were identical over all regions of overlap; however, there was a 96-bp region for which there were three different but homologous variants (A, B, and F). This region of divergence was identified as an alternatively spliced cassette exon since clones were identified that contained intronic DNA as well as consensus splice acceptor sites that bounded the region. Tissue Northern blot analysis revealed a broad band at ≈5.1 kb that was unique to the kidney. High-stringency Northern blot analysis of cortical and medullary mRNA using antisense oligonucleotides synthesized over each of the three cassette exons revealed that the isoforms were differentially distributed within the kidney—B almost exclusively in cortex, F almost exclusively in medulla, and A about equally distributed.**

The ability of the mammalian kidney to produce a concentrated urine is dependent largely on the transport properties of the thick ascending limb of the loop of Henle (TAL) in which NaCl is reabsorbed in excess water. Early micropuncture studies demonstrated that fluid emerging from the TAL was dilute (1, 2) and subsequent studies using *in vitro* microperfused TAL segments helped define the cellular transport process as active Cl absorption dependent on the basolateral Na pump (3, 4). It is now known that reabsorption of salt in the TAL requires the activity of an apical cotransporter, which uses the inward gradient of Na generated by the basolateral Na pump, to move Na, K, and Cl in an electrically neutral fashion (5). As specific inhibitors of the Na-K-Cl cotransporter, the clinically important "loop" diuretic drugs furosemide and bumetanide block the last major site of Na reabsorption in the nephron and cause salt and water loss.

The Na-K-Cl cotransporter has been identified as a [^{3}H]-bumetanide-binding protein in mammalian kidney (6) and numerous other tissues (7, 8). Although there has been little progress in purification of the protein from kidney, photoaffinity labeling studies using an analog of bumetanide have identified a 150-kDa glycosylated protein as at least part of the cotransporter (9). Recently, we cloned and expressed a cDNA encoding the basolateral form of the bumetanide-sensitive Na-K-Cl cotransporter from a model secretory epithelium, the shark rectal gland (10). On Northern blot analysis, this cotransporter is encoded by a widely distributed 7.4-kb message in shark tissues. We also identified an abundant ≈5.2-kb message that was unique to shark kidney. Physiological and immunolocalization studies (11, 12) show that in the shark, as in the mammal, the Na-K-Cl cotransporter is predominantly an apical membrane protein in the kidney, whereas it has a basolateral distribution in secretory epithelia. These findings, as well as differences in protein molecular weights (see ref. 13), diuretic affinities (14), and antibody recognition (12, 15), suggest that the absorptive form of the Na-K-Cl cotransporter in the kidney is a distinct isoform, possibly encoded by a separate gene. In this report, we present the cloning and sequencing of a cDNA from rabbit kidney, which is proposed to encode the renal absorptive form of the Na-K-Cl cotransporter. Three alternatively spliced variants of this gene have been identified and were found to display a distinct differential distribution in the kidney cortex and medulla.*

MATERIALS AND METHODS

Cloning and Sequence Analysis. Probes were obtained from a human colonic (T84) cDNA library by screening with shark rectal gland Na-K-Cl cotransporter cDNAs (J. C. Xu, J.A.P. and B.F., unpublished data). Two nonoverlapping clones, encoding the transmembrane region of the T84 Na-K-Cl cotransporter (J.A.P., J. C. Xu, and B.F., unpublished data), were obtained; these displayed 55% and 75% identity to the shark cotransporter (−107–747 and 988–2194 nt). The T84 cDNAs were labeled with ^{32}P by random priming and used to screen two rabbit kidney libraries (cortex and medulla in λZAP; gifts from W. Guggino, Johns Hopkins University) under low-stringency conditions [30°C in 50% formamide/5× standard saline phosphate/EDTA (SSPE)/5× Denhardt's solution/0.1% SDS]. The cloned cDNA inserts were sequenced bidirectionally by the dideoxynucleotide chain-termination method (16) using a combination of manual sequencing with Sequenase II (United States Biochemical) and automated sequencing (Applied Biosystems) with synthetic oligonucleotide primers and fluorescent dideoxynucleotide terminators. The program TBLASTN (17) was used to search GenBank. Identity measurements between similar sequences are expressed as the percentage of amino acids in the first sequence that are matched in the second sequence after optimal alignment with minimal gaps.

Northern Blot Analysis. Total RNA was isolated from fresh rabbit tissues, spiny dogfish (*Squalus acanthias*) rectal gland, and T84 cultured cells by the guanidine thiocyanate method (18). Poly(A)$^+$ RNA was purified from total RNA by using magnetic beads (PolyATtract; Promega). Poly(A)$^+$ selected RNA was denatured by heating to 65°C in formamide and formaldehyde and size-fractionated on a 1% agarose gel. The fractionated mRNA was transferred to a nylon membrane by semidry blotting. The following hybridization conditions were

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Abbreviations: TAL, thick ascending limb of the loop of Henle; TSC, thiazide-sensitive Na–Cl cotransporter.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U07547, U07548, and U07549).

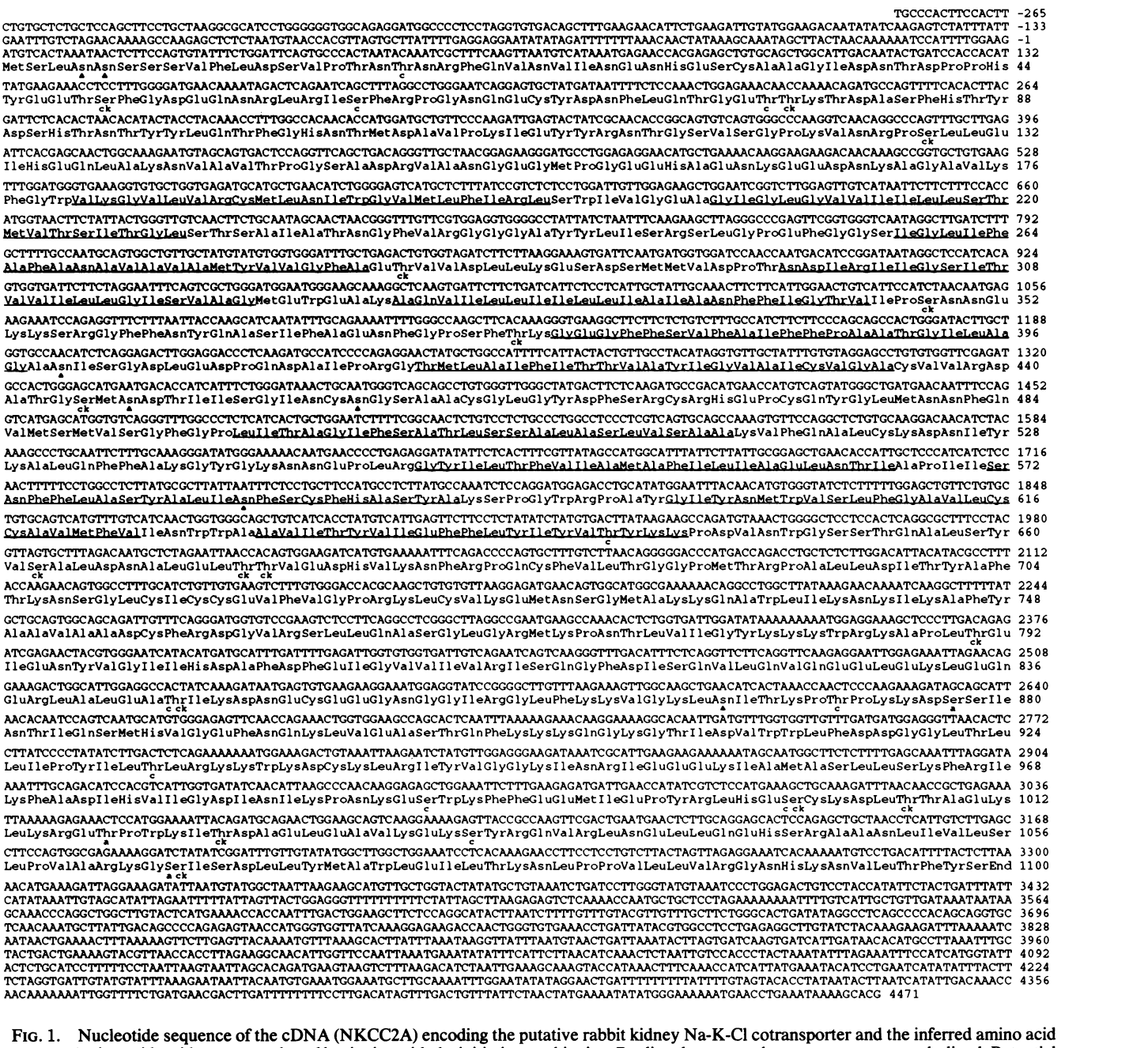

FIG. 1. Nucleotide sequence of the cDNA (NKCC2A) encoding the putative rabbit kidney Na-K-Cl cotransporter and the inferred amino acid sequence. Amino acid residues are numbered beginning with the initiation methionine. Predicted transmembrane segments are underlined. Potential sites of N-linked glycosylation (▲), and consensus targets for protein kinase A (a), protein kinase C (c), and casein kinase II (ck) are labeled.

used: (*i*) cRNA probe—24 h at 45°C in 50% formamide/2× SSPE/2× Denhardt's solution/1% SDS/200 μg of yeast RNA per ml/100 μg of fish sperm DNA per ml; (*ii*) synthetic 84-bp oligonucleotides—4 h at 68°C in 5× SSPE/0.5% SDS/10× Denhardt's solution/100 μg of fish sperm DNA per ml; (*iii*) control cDNA probe—8 h at 65°C in 0.25 M Na_2HPO_4, pH 7.2/1 mM EDTA/7% SDS. Membranes were prehybridized for 2–4 h and then hybridized in fresh solution containing 10^6 cpm of ^{32}P-labeled probe per ml. The antisense cRNA probe was produced as a run-off transcript (MAXIscript; Ambion, Austin, TX) from one of the cDNAs (clone M31f; 885–1385 nt). To identify the distribution of the isoforms within the kidney, single-stranded antisense oligonucleotides were synthesized over the region of highest divergence in the cassette exon (84 bp; 637–720 nt; identity: A and B, 60%; A and F, 50%; B and F, 64%). These oligonucleotides, made for each of the three splice isoforms, were gel purified and end labeled with ^{32}P and polynucleotide kinase. A control cDNA probe coding for a conserved region of the sequence (885–2250 nt) was ^{32}P labeled by random priming. The membranes were subjected to a final wash depending on the probe used: (*i*) cRNA probe, 20 min at 65°C in 0.1× standard saline citrate (SSC)/0.5% SDS; (*ii*) synthetic 84-bp oligonucleotides, 15 min at 37°C in 5× SSPE/0.5% SDS; (*iii*) control cDNA probe, 15 min at 37°C in 40 mM Na_2HPO_4, pH 7.2/1 mM EDTA/1% SDS. To reprobe a membrane, the blot was stripped by a 1-h incubation at 72°C in 0.1× SSC/0.5% SDS, and probe removal was verified by autoradiography at −70°C with an intensifying screen for 24 h.

RESULTS AND DISCUSSION

To obtain rabbit renal cDNAs encoding the Na-K-Cl cotransporter, we screened both a cortical and a medullary kidney library by using two nonoverlapping fragments of the secre-

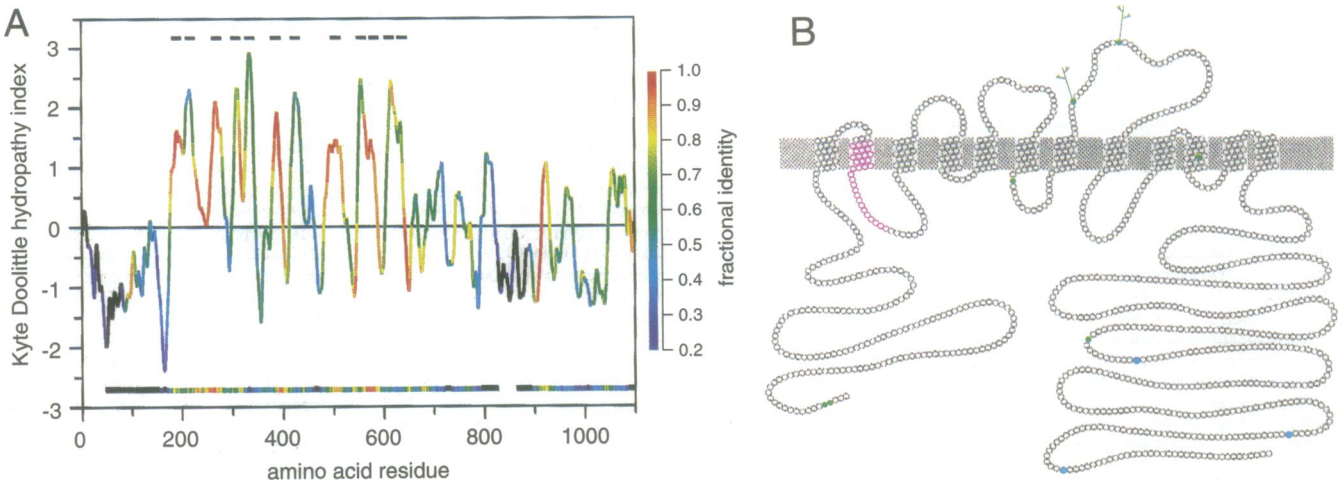

FIG. 2. (*A*) Hydropathy profile of the putative absorptive Na-K-Cl cotransporter from kidney (NKCC2). The hydropathy index was determined by the Kyte and Doolittle algorithm (20) using a 15-amino acid window. The hydropathy line is color coded by the fractional identity of NKCC2 to the secretory Na-K-Cl cotransporter from shark rectal gland (NKCC1) averaged over a running 15-amino acid window after aligning the two sequences (see text). The color-coded line at the bottom of the graph indicates identity with the TSC after similar alignment (larger gaps are indicated by white space). Horizontal bars above hydrophobic regions indicate the proposed transmembrane segments. (*B*) Proposed model of the putative renal Na-K-Cl cotransporter. Circles symbolize amino acid residues. The region highlighted in red is encoded by an alternatively spliced cassette exon. Potential sites for N-linked glycosylation are highlighted in green; branched lines specify those sites presumed to be capable of anchoring oligosaccharide. Consensus sites for phosphorylation are highlighted in blue.

tory Na-K-Cl cotransporter (see *Materials and Methods*). Forty-two cDNAs were plaque purified and excised into Bluescript SK− (10 from cortex and 32 from medulla). Each cDNA was characterized by restriction enzyme analysis, Southern blotting, and 5′- and 3′-end sequencing. Among six overlapping clones that were sequenced in full (see Fig. 4 below), all were identical at the nucleotide level except for a short 96-bp region (discussed below), a piece of intronic DNA (clone C24a), and several single base changes that were presumably due to library construction artifacts or allelic variation (19). An overlapping nucleotide sequence of 4750 bp was thus identified (NKCC2; Fig. 1).

The NKCC2 cDNA includes a full-length open reading frame encoding 1099 amino acids, beginning with the first ATG downstream of a stop codon and followed by a polyadenylylation signal sequence (AATAAA) 6 bases upstream of the end of the sequence. The predicted molecular mass is 121 kDa, which is in the range expected for a core polypeptide corresponding to the glycosylated renal cotransporter identified by photoaffinity labeling (150 kDa; ref. 9). The amino acid sequence can be aligned exactly with that of the shark rectal gland Na-K-Cl cotransporter (NKCC1; ref. 10) with only four small gaps: 1- and 5-residue insertions (at residues 350 and 141), and 1- and 12-residue deletions (at residues 456 and 851), following an 80-residue truncation of the N terminus. Over the full length, the predicted kidney protein is 61% identical to the secretory cotransport protein, suggesting that NKCC2 encodes the absorptive form of the cotransporter found in the renal tubule.

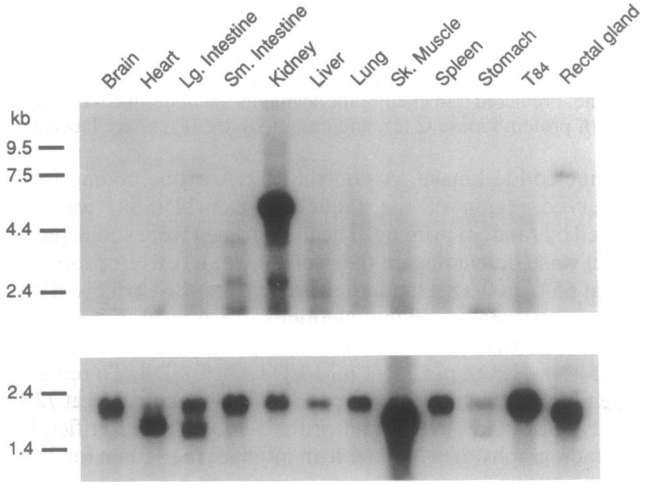

FIG. 3. Northern blot analysis of expression of NKCC2 and related mRNAs in rabbit tissues, T84 cells, and shark rectal gland. (*Upper*) mRNA (5–10 μg) was hybridized with a 500-bp antisense cRNA probe transcribed *in vitro* from clone M31f. (*Lower*) Blot was reprobed with a β-actin cDNA probe as a control for RNA integrity.

The hydropathy analysis of NKCC2 (Fig. 2*A*) demonstrates a large central hydrophobic region bounded by N- and C-terminal hydrophilic domains—very similar to the profiles of the NKCC1 and the thiazide-sensitive Na-Cl cotransporter from flounder urinary bladder (TSC; ref. 21). We presume that the N and C termini are cytoplasmic, based on homology with the corresponding regions in NKCC1. Our current interpretation of the hydropathy plot modeled in Fig. 2*B* thus includes 12 transmembrane helices in the central region (indicated as bars in Fig. 2*A*). The polypeptide sequence of NKCC2 has 7 potential N-linked glycosylation sites (Fig. 1). In the proposed model, 2 of these sites are located in an extracellular hydrophilic region of the protein between putative transmembrane segments 7 and 8.

The degree of identity between NKCC2 and the secretory Na-K-Cl cotransporter is illustrated by the color coding of the hydropathy plot in Fig. 2*A*. In addition, the bottom line in Fig. 2*A* shows the homology with TSC, which displays 45% identity over the full length. Overall, the hydrophobic regions display the greatest similarity among these proteins, with 57–100% (NKCC2 to NKCC1) or 29–91% (NKCC2 to TSC) identity seen in the putative transmembrane domains. Predicted transmembrane segments 1, 3, 6, 8, and 10 all show >91% identity between NKCC1 and NKCC2. At least two regions that are predicted to be outside of the membrane are highly conserved—these include the loop connecting helices 2 and 3, and a short region in the center of the C-terminal hydrophilic domain. There is also significant homology to cyanobacterial (22) and *Caenorhabditis elegans* (23, 24) sequences, as previously discussed for NKCC1 (10).

Vasopressin, acting through adenylate cyclase, is known to increase reabsorption of NaCl across the TAL of some mam-

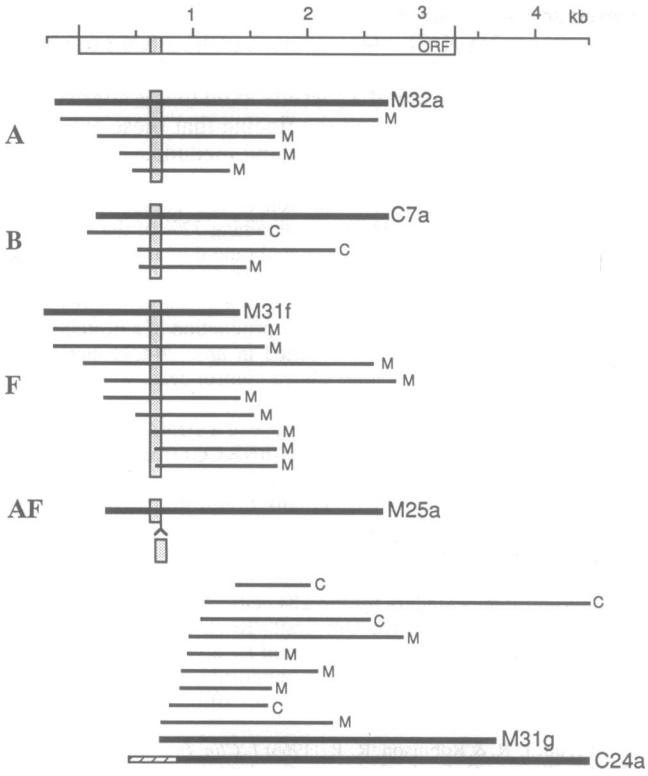

FIG. 4. Schematic diagram of clones isolated from renal libraries. Clones isolated from the medullary library are denoted M; those from the cortical library are denoted C. Fully sequenced clones are indicated by heavier lines. All clones were sequenced over the region of the ABF cassette exon (stippled bars). Eleven clones (bottom group) were too short to contain the ABF cassette exon. The 3297-nucleotide open reading frame (ORF) is indicated by the box at the top. Clone C24a was found to contain a piece of intronic DNA (hatched bar). Eleven other clones that apparently contained intronic DNA (or foreign DNA resulting from cloning artifacts) are not shown here.

malian species, apparently by activating apical Na-K-Cl cotransporters (see ref. 25). There are three potential sites for phosphorylation of NKCC2 by cAMP-dependent protein kinase located within the cytoplasmic C-terminal region (Ser^{878}, Thr^{1017}, and Ser^{1064}; Figs. 1 and 2). This is in contrast to the secretory cotransporter, which does not have consensus protein kinase A sites (10). Interestingly, the sequence Arg-Glu-Thr^{1017} in NKCC2 corresponds to a sequence with three constitutive negative charges in NKCC1, Glu-Glu-Glu^{1109}; these charges could be expected to alleviate a requirement for phosphorylation at this position.

Our efforts to achieve functional expression of NKCC2 have been unsuccessful. We have prepared full-length constructs of NKCC2 (A, B, and F variants; see below) in the expression vector pJB20 and have attempted to express these in HEK-293 and COS cells. Although previous (10) and parallel efforts (J.A.P., J. C. Xu, and B.F., unpublished data) with secretory forms of the Na-K-Cl cotransporter were positive, transfection with NKCC2 did not lead to increased total or bumetanide-sensitive ^{22}Na or ^{86}Rb influxes, nor was immunoreactive protein produced by the cells. When truncated constructs (initiating at Met^{105}) were prepared, immunoreactive proteins of the expected size were observed (≈105 kDa) after transient transfection of COS cells (data not shown). The truncated proteins were apparently not glycosylated, indicating that they may not have been correctly folded. We speculate that the failure to achieve detectable levels of expression of full-length NKCC2 is due to problems with mRNA stability or initiation of translation. Difficulty in expression of particular membrane proteins in foreign cells has been reported (26).

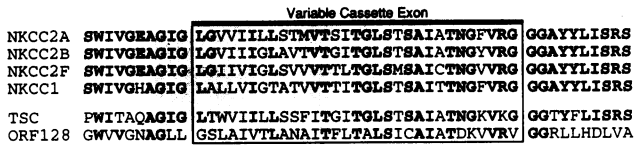

FIG. 5. Amino acid sequence alignment of the ABF cassette region. A, B, and F variants (NKCC2A, -B, -F; see text) are compared to the sequences from the secretory Na-K-Cl cotransporter (NKCC1), the TSC, and a translated open reading frame from cyanobacterium (ORF128). Conserved residues are in boldface type. The extent of the variable cassette is boxed and the position of the second putative transmembrane domain is shaded.

To determine the tissue expression of NKCC2 and related mRNAs, we examined a Northern blot containing poly(A)$^+$ RNA from several rabbit tissues, T84 cells, and shark rectal gland. Using an antisense cRNA probe encoding a conserved region of NKCC2, a broad band at ≈5.1 kb was expressed at a very high level in rabbit kidney and was specific to the kidney (Fig. 3). The only other transcript detectable at this exposure was a 7.4-kb message in shark rectal gland, which corresponds to the Na-K-Cl cotransporter mRNA transcript, previously identified in this tissue (10). Upon longer development, a transcript was detected between 7.0 and 7.5 kb in large intestine, stomach, and T84 cells (data not shown). This latter transcript was detected at a high level with a cRNA probe made from the human colonic Na-K-Cl cotransporter (J.A.P., J. C. Xu, and B.F., unpublished data), and it is clear that it represents the message for the secretory cotransporter.

The identification of an ≈5.1-kb message, which was specific to renal tissue, is consistent with our results from shark tissue Northern blot analysis of the rectal gland Na-K-Cl cotransporter, which demonstrated a unique ≈5.2-kb message in the kidney (10). Both the similar message size and the fact that the transcripts are unique to renal tissue in both species provide further support for the hypothesis that the cDNA we have isolated from rabbit kidney encodes the absorptive form of the Na-K-Cl cotransporter.

In characterizing the 42 clones isolated from the renal cDNA libraries we noted that a single 96-bp region of sequence was markedly divergent among different clones (Fig. 4). We sequenced each of the cDNAs over this region and found that three alternative sequences were represented, termed types A, B, and F. Several cDNAs were also identified, which were produced from unprocessed mRNA during library construction—two of these clones contained consensus splice acceptor sites (27) clearly identifying the 96-bp region as an alternatively spliced exon (M48b, gcatttgcag-GTCTTGGA; M31a, tctgtt-

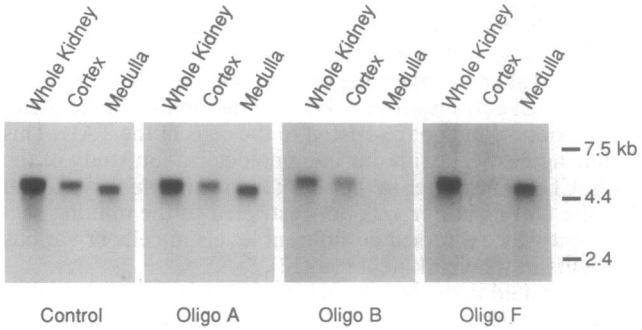

FIG. 6. Northern blot analysis of the expression of the ABF cassette variants in rabbit renal cortex and medulla. mRNA was prepared from dissected rabbit kidneys, discarding the boundary region between the dark red medulla and the cortex. The same blot (5 μg of mRNA in each lane) was successively hybridized with a control cDNA from NKCC2 and with single-stranded antisense 84-bp oligonucleotides synthesized over each of the cassette exons—A, B, and F.

tcag-GTGGGGCC; data not shown). Thus, it is evident that A, B, and F cDNAs are alternatively spliced variants of NKCC2.

The alternatively spliced cassette exons of NKCC2 encode most of putative transmembrane segment 2 as well as 13 amino acids located intracellularly in our model (Fig. 2*B*). Fig. 5 displays the amino acid comparison for the region around the cassette exon for the splice variants and for NKCC1, TSC, and for a homologous cyanobacterial open reading frame (22). Within the cassette exon, the splice variants exhibit no significantly greater identity to each other (69%) than to NKCC1 (67%) or TSC (65%). Putative transmembrane segment 2 shows various hydrophobic amino acid substitutions among the proteins. Interestingly, the putative cyanobacterial protein contains residues that are well conserved among the different cotransporters. The amino acids between helices 2 and 3 are particularly well conserved; however, 3 variant residues occur at a regular interval, suggesting a role of one face of an α-helical structure. Also notable within the cassette exon is the preponderance of serine and threonine residues.

It appears important that the alternatively spliced ABF cassette exons encode a transmembrane segment of the protein and that there is strict conservation of about half the residues as well as regularly spaced variability at the 3′ end of the exon. It is possible that the peptide encoded in this region forms part of the ion translocation pocket and that the variants exhibit different ion affinities or even different ion specificities. It is intriguing to note that K-independent bumetanide-sensitive Na-Cl cotransport has been described in the mouse TAL and that its regulation by vasopressin is reciprocal to that of the K-dependent Na-Cl cotransporter (28).

A single medullary cDNA contained both A and F cassettes in tandem (Fig. 4). Since the ABF cassette exons encode a topologically important portion of the protein (i.e., putative transmembrane segment 2), one would expect them to be differentially spliced in a mutually exclusive fashion. Thus, it seems most likely that the AF tandem construction found in a single cDNA is an artifact of incomplete splicing and would not produce a functional protein.

The distribution of the NKCC2 variants in the rabbit kidney was obtained by isolating poly(A)$^+$ RNA from kidney cortex and medulla and hybridizing at high stringency with synthetic oligonucleotides corresponding to the most divergent region of the ABF cassettes (Fig. 6). Whereas variant A was distributed in both cortex and medulla, variant B was restricted to the cortex and variant F was found principally in the medulla. This distribution of the variants was also supported by the origin of the cDNAs: clones isolated from the cortical library that coded over the cassette exon were all of the B form, whereas clones from the medullary library were predominantly of the F form (Fig. 4). The medullary location of the F form transcript and the high percentage of these cDNAs suggest that this variant encodes the putative Na-K-Cl cotransporter from the medullary TAL and is responsible for NaCl reabsorption in that segment. Taken together, the observations above suggest that the Na-K-Cl cotransporter is broadly distributed in the mammalian kidney and not restricted to the cells of the TAL. This finding agrees well with our immunolocalization study of the Na-K-Cl cotransporter in the shark kidney in which we found that the cotransporter was not restricted to the diluting segment but was expressed at different levels in cells of various segments in the shark nephron (12).

It was noted in the Northern blot shown in Fig. 6 as well as in other similar experiments that the cortical transcript was $\approx$200 bp larger than the medullary transcript. This observation suggests that there are regional differences in RNA processing (e.g., polyadenylylation). At this time, it is unclear whether the transcript size difference is directly related to the differential distribution of the splice variants.

The above results provide strong evidence that the NKCC2 cDNA encodes the renal isoform of the Na-K-Cl cotransporter: the newly described cDNA is 61% identical to that of the secretory Na-K-Cl cotransporter and the mRNA transcript appears to be found exclusively in the kidney. We also report the intriguing discovery of a cassette exon that is alternatively spliced in three variants and the finding that these are differentially expressed in renal cortex and medulla.

Note Added in Proof. After submission of this paper, we obtained functional expression of a chimera containing 72% of the rNKCC2a sequence, including all of the transmembrane domains and the large C terminus. The construct was composed of Met^1–Thr^{218} of hNKCC1 (29) and Met^{105}–Ser^{1099} of rNKCC2a. When stably expressed in HEK-293 cells, the cDNA directed the production of a protein of the expected size (~165 kDa) and resulted in an ~20-fold increase in bumetanide-sensitive ^{86}Rb influx above control cells.

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