Cloning, characterization, and gene organization of K-Cl cotransporter from pig and human kidney and *C. elegans*

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Holtzman, Eli J., Sumit Kumar, Carol A. Faaland, Fern Warner, Paul J. Logue, Sara J. Erickson, Gesa Ricken, Jeremy Waldman, Shiv Kumar, and Philip B. Dunham. Cloning, characterization, and gene organization of K-Cl cotransporter from pig and human kidney and *C. elegans*. *Am. J. Physiol.* 275 (*Renal Physiol.* 44): F550–F564, 1998.—We isolated and characterized the cDNAs for the human, pig, and *Caenorhabditis elegans* K-Cl cotransporters. The pig and human homologs are 94% identical and contain 1,085 and 1,086 amino acids, respectively. The deduced protein of the *C. elegans* K-Cl cotransporter clone (CE-KCC1) contains 1,003 amino acids. The mammalian K-Cl cotransporters share ~45% similarity with CE-KCC1. Hydropathy analyses of the three clones indicate typical KCC topology patterns with 12 transmembrane segments, large extracellular loops between transmembrane domains 5 and 6 (unique to KCC), and large COOH-terminal domains. Human KCC1 is widely expressed among various tissues. This KCC1 gene spans 23 kb and is organized in 24 exons, whereas the CE-KCC1 gene spans 3.5 kb and contains 10 exons. Transiently and stably transfected human embryonic kidney cells (HEK-293) expressing the human, pig, and *C. elegans* K-Cl cotransporter fulfilled two (pig) or five (human and *C. elegans*) criteria for increased expression of the K-Cl cotransporter. The criteria employed were basal K-Cl cotransport; stimulation of cotransport by swelling, *N*-ethylmaleimide, staurosporine, and reduced cell Mg concentration; and secondary stimulation of Na-K-Cl cotransport.

inorganic ion cotransport; cell volume regulation; HEK cells; transient and stable transfection

REGULATION OF CELL VOLUME is a fundamental property of all cells. Animal cells contain an excess of impermeant solute compared with the extracellular fluid. To maintain constant volume, cells must expend energy through primary and secondary active transport mechanisms to prevent cell swelling. Many transporters play roles in cell volume regulation (15). Epithelial cells involved in transcellular fluid transport present a special problem: the rate of water influx on one side of the cell and efflux on the other side must be precisely equal (32).

Activation of K-Cl cotransport by cell swelling can play a role in the regulation of cell volume by promoting K-Cl efflux accompanied by an osmotically obliged efflux of water. Early evidence of K-Cl cotransport was obtained from red blood cells from sheep of the low cell K concentration (LK) phenotype, in which it was observed as a Cl-dependent K flux that was particularly sensitive to osmotically induced increases in cell volume (7). K-Cl cotransport is elevated in red blood cells from patients with sickle cell anemia and may be important in the pathogenesis of this condition (1, 17).

The K-Cl cotransporter (KCC) is a member of a family of inorganic cation-chloride cotransporters that also includes the Na-K-Cl cotransporter (NKCC) and the Na-Cl cotransporter (NCC). Several years ago, the structures of NKCC (6, 10, 22) and NCC (11) were established. More recently, the molecular cloning of the K-Cl cotransporters by Gillen et al. (13), Payne et al. (27), and us (19) has established the structure of the K-Cl cotransporter (KCC), the third member of the family. Two isoforms of KCC have been identified, one being ubiquitous (KCC1) (13, 19) and the other neuronal (KCC2) (25, 27).

The human K-Cl cotransporter cloned by Gillen et al. (13) and by us (19) is from kidney, but the cell type from which it came is unknown. We cloned the K-Cl cotransporter from a cDNA library from LLC-PK_1 cells, a cell line derived from pig nephron.

The human K-Cl cotransporter gene was found at a locus in chromosome 16q22.1, which contains five genes within 40 kb of genomic DNA (9, 13). The genes code for the putative proteasome subunit MECL1, chymotrypsin-like protease (CTRL), a protein serine kinase (PSKH1), lecithin:cholesterol acyl transferase (LCAT), and the K-Cl cotransporter.

The database from the *Caenorhabditis elegans* Sequencing Consortium allowed identification of two genes that appeared to code for K-Cl cotransporters. We cloned the cDNA from one of these genes (CE-KCC1), expressed it in mammalian cells, and characterized the function of the protein. It is a K-Cl cotransporter with functional properties similar to the mammalian KCC1. *C. elegans* is the lowest organism from which a K-Cl cotransporter, or any member of the cation-chloride cotransporter family, has been characterized. A cation-chloride cotransporter has also been cloned from the tobacco plant (14), and evidence of a functional role for the COOH terminus was presented. A cation-chloride cotransporter was also isolated from the insect *Manduca sexta* (31), but has not been characterized functionally.

Defining the structure and the function of the K-Cl cotransporters will permit the study of structure-

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function relationships of this cotransporter and among the three members of the family. Results of these studies will lead to new approaches to the study of the regulation of K-Cl cotransport and its roles in cell function, particularly in the control of cell volume.

METHODS

Cloning of the human K-Cl cotransporter (KCC1). In the Expressed Sequence Tag database (EST), there are multiple clones with limited tag sequences that had ~30% similarity to Na-K-Cl and Na-Cl cotransporters. These clones could be divided into two groups. The sequence of one group (ID numbers 133908, 153271, 154265, and 159563) had significant homology to the sixth transmembrane domain in the cotransporter family. The second group (ID numbers 67110, 152542, 154000, 154505, 156182, and 182942) showed similarity to the amino acid sequence in the COOH-terminal tails of the known cotransporters. Restriction analysis, further sequencing, and polymerase chain reaction (PCR) amplification showed that all these clones are part of a single transcript. A fragment amplified by PCR was used as a probe to screen a human kidney cDNA library. The forward primer was 5′-ATCTTCTTCCCTTCTGTAACAGGCATCATG-3′, and the reverse primer was 5′-TGCTCTAGATCAGGAGTAGATGGTGATGACTTCACG-3′.

A λGT11 human kidney cDNA library (Clontech, Palo Alto, CA) was screened. Plaques hybridizing to the probe were isolated by limiting dilution and were rescreened to homogeneity. One positive cDNA clone was obtained, and its purified insert was subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA). This clone lacked the 5′ coding region of the K-Cl cotransporter.

To clone the missing 5′ segment, a 5′ rapid amplification of cDNA ends (RACE) of a human kidney marathon-ready cDNA (Clontech) was performed. PCR was conducted according to the manufacturer's instructions, using one outer reverse gene-specific primer (GSP1) (5′-CCCTGTGGGGACCATTCTGGCCATCATTAC-3′) from the most 5′ area of the cloned piece and an adapter primer (AP1) (5′-CCATCCTAATAGCACTCACTATAGGGC-3′) (Clontech). This was followed by a nested PCR using the nested gene-specific primer (GSP2) (5′-GTAATGATGGCCAGAATGGTCCCCACAGGG-3′) and a nested adapter primer (AP2) (5′-ACTCACTATAGGGCTCGAGCGGC-3′) (Clontech). In this way, we were able to clone the most 5′ coding area, including the ATG start site. This fragment was subcloned, sequenced, and ligated to the previously cloned library-based truncated K-Cl cotransporter cDNA piece using a unique *Tth* III 1 restriction site.

Cloning of the pig K-Cl (KCC1) cotransporter from a LLC-PK$_1$ cell library. We identified two human K-Cl cotransporter primers (forward, 5′-ATCTTCTTCCCTTCTGTAACAGGCATCATG-3′; reverse, 5′-TGCTCTAGATCAGGAGTAGATGGTGATGACTTCACG-3′) that, under moderate-stringency annealing conditions, amplified a similar-length fragment (2.1 kb) from a cDNA pool of 500,000 clones of the LLC-PK$_1$ plasmid library. This fragment was sequenced and found to be homologous to the human K-Cl cotransporter, suggesting the presence of a pig K-Cl cotransporter transcript in the tested pool. Miniprep DNA from pooled colonies of bacteria was used as a template for PCR with the primers noted above. PCR conditions were 94°C for 1 min, followed by 42 cycles with the following temperature program: 94°C for 30 s, 48°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 7 min.

This procedure was repeated using cDNA extracted from 12 pools of 50,000 clones and then was scaled down by 10 steps through the use of the positive glycerol stocks for growing the 10-fold smaller cDNA pools. At the end of this PCR cloning procedure, one clone that contained the full-length pig K-Cl cotransporter was obtained.

Cloning of the C. elegans K-Cl cotransporter. Cloning of the *C. elegans* K-Cl cotransporter (CE-KCC1) was done by PCR of a cDNA library in λGT11 derived from mixed-stage *C. elegans* hermaphrodites (strain N2), a gift from Drs. Peter Okkema and Andrew Fire (23). The library was diluted (1:20) with distilled water and incubated for 20 min at 70°C. The sample was then spun, and 1 μl of the supernatant was used as a DNA template for the PCR reaction.

Primer selection was based on the CE-KCC1 genomic sequence (GenBank accession no. U40798). The forward ATG primer was 5′-ATGCCATTTTTCTCTAGCTATCTGAAAGCCCATATC-3′, and the reverse primer was 5′-TTACGAGCTCTCCGTGATCACTTCTTTGCCAGTTCC-3′. PCR conditions were 94°C for 1 min, followed by 42 cycles with a temperature program of 94°C for 30 s, 48°C for 30 s, and 72°C for 2 min; followed by a final extension at 72°C for 7 min using High Fidelity Expansion *Taq* polymerase (Boehringer, Indianapolis, IN). The amplified 3-kb fragment was subcloned to the eukaryotic expression vector pCR3 (Invitrogen, Carlsbad, CA) using the eukaryotic bidirectional TA cloning kit (Invitrogen). Both strands of the clones were sequenced (T7 Sequenase version 2.0; Amersham, Arlington Heights, IL) to exclude the possibility of PCR mutations. One cDNA clone, which was confirmed to be wild type by comparison with the genomic sequence published in GenBank, was used for functional studies.

Secondary structure predictions and sequence analysis. Sequence analysis and alignments were done using EDITSEQ and MEGALIGN sequence analysis software (Lasergene; DNASTAR, Madison, WI). Similarity and identity of various KCC clones were determined by using the National Center for Biotechnology Information (NCBI) BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and by alignment using the MEGALIGN sequence analysis software.

The phylogenetic tree was constructed by the unweighted pair-group method with arithmetic mean using MEGALIGN sequence analysis software. A probable model for transmembrane topology was generated using three programs: SOSUI program [Tokyo University of Agriculture and Technology (http://www.tuat.ac.jp/~mitaku/adv_sosui/)], PhdTopology Predict-Protein Program [EMBL-Hiedelberg, Germany (http://www.embl-heidelberg.de/predictprotein/predictprotein.html)] and TMpred [prediction of transmembrane regions and orientation program (http://ulrec3.unil.ch/software/TMPRED_form.html)]. Hydropathy plots were made using the Kyte-Doolittle algorithm with a window of 12 amino acid residues (20). Prediction of hydropathy was made using DNA Strider 1.2 software.

PCR analysis for the presence of the K-Cl cotransporter cDNA was done by amplification of various human cDNA libraries with the 3′ coding region K-Cl cotransporter using forward primer 5′-ATCTTCTTCCCTTCTGTAACAGGCATCATG-3′ and reverse primer 5′-TGCTCTAGATCAGGAGTAGATGGTGATGACTTCACG-3′.

Human gene organization analysis. Human gene organization data were obtained as follows. The most downstream seven exons and introns were obtained by comparison of cDNA sequences we had cloned with the human genomic sequences in the database (GenBank accession no. X51966). Additional gene organization data, which included the most 5′ 17 exons and introns, were obtained using long PCR (Expand Long Template PCR System; Boehringer Mannheim) of normal human genomic DNA followed by DNA sequencing and comparison to the cloned human cDNA sequence.

C. elegans gene organization analysis. Chromosomal localization and gene organization were analyzed using data from GenBank accession no. U40798. Our CE-KCC1 cDNA sequence was compared with the *C. elegans* genomic sequence data, and intron-exon boundaries were defined.

Transfection of HEK-293 cells. Human embryonic kidney cells (HEK-293) were grown to confluence in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum and penicillin and streptomycin, at 37°C in a humidified atmosphere with 5% CO_2.

The full-length K-Cl cotransporter cDNA constructs from the human and pig were subcloned to the eukaryotic expression vector pCDNA3 (Invitrogen). The *C. elegans* cDNA was subcloned to the eukaryotic expression vector pCR3 (Invitrogen).

For transient expression, plasmids were transfected into HEK-293 cells by calcium phosphate precipitation (16). Optimal transfection efficiency was obtained by the addition of 20 μg of total plasmid DNA to a 55-cm^2 plate (Falcon, Lincoln Park, NJ) followed by incubation for 20 h without glycerol shock. Cells were then trypsinized and transferred, at a density of 2×10^5 cells/well, to a 24-well plate for another 24 h until assayed. The wells had been coated with collagen or poly-D-lysine (Fisher Scientific) to promote adherence of the cells.

For stable expression, HEK-293 cells containing stably integrated plasmids derived from the human K-Cl cotransporter were selected by resistance to 1.0 mg/ml Geneticin (G418; Life Technologies, Gaithersburg, MD). Stably transfected cell lines were maintained chronically with 0.5 mg/ml Geneticin. Clonal cells were trypsinized and transferred, at a density of 1.5×10^5 cells/well, to a 24-well plate for another 24 h until assayed. Stable integration of the human K-Cl cotransporter cDNA was confirmed by the presence of a PCR fragment derived from the cDNA that did not contain introns. The forward primer was 5′-ATCTTCTTCCCTTCTGTAACAGGCATCATG-3′, and the reverse primer was 5′-TGCTCTAGATCAGGAGTAGATGGTGATGACTTCACG-3′.

Stable integration of the *C. elegans* cDNA was confirmed by PCR of genomic DNA from HEK-293 cells. The forward primer was 5′-ATGCCATTTTTCTCTAGCTATCTGAAAGCCCATATC-3′, and the reverse primer was 5′-TTACGAGCTCTCCGTGATCACTTCTTTGCCAGTTCC-3′.

Assay of K-Cl cotransport in HEK-293 cells. Unidirectional K influxes were measured at 26°C using ^{86}Rb as a tracer; Rb is a good congener for K in K-Cl cotransport (7). Cells were transferred to 24-well plates, incubated overnight, and allowed to reach ~50% confluence. The growth medium was removed by aspiration and replaced with 0.5 ml of standard medium containing (unless otherwise specified) (in mM) 135 NaCl, 5 KCl, 0.75 $MgSO_4$, 0.75 $CaCl_2$, 0.1 ouabain, and 15 HEPES brought to pH 7.4 with Tris base. In the experiments on *C. elegans* clones, the cells were allowed to preequilibrate in the HEPES-buffered medium for 60 min at 26°C in air before we started the pretreatments and flux assays. This was done to ensure that the cells fully recovered from the pH increase that can occur as the cells are switched from their growth medium with 5% CO_2 to a HEPES-buffered medium in air.

The desired agents were added after preincubation in standard medium, usually for 10 min. As desired, preincubation medium contained 10–30 μM bumetanide, which inhibited all Na-K-Cl cotransport (2 μM inhibited ~90%). Preincubation medium was removed by aspiration, and then 0.5 ml of standard medium containing 2 μCi of ^{86}Rb (Cl salt) was added. Triplicate wells were set up for each condition. The flux period was 3–4 min. Preliminary experiments had shown that uptake of tracer is linear for at least 7 min, both in control and transfected cells. Flux medium (standard medium + ^{86}Rb) was removed by aspiration, and the cells were washed free of extracellular tracer by three quick rinses with ice-cold, tracer-free, standard medium. Cells were removed from the wells by incubation for 30 min at 37°C with 1 ml of 1% SDS. The radioactivity of each 1 ml cell extract and of a 50-μl sample of the flux medium was determined in a liquid scintillation counter by Cerenkov radiation. The protein concentration of each sample was then determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). The K influxes are expressed ± SD as nanomoles per milligram protein per minute.

In most experiments on human and pig clones, K-Cl cotransport was defined as the Cl-dependent K influx in the presence of 10 μM bumetanide, with gluconate used as the substitute anion for Cl. In one experiment, the criterion for K-Cl cotransport was the bumetanide-insensitive K influx inhibited by 2 mM furosemide. In later experiments on *C. elegans* clones, K-Cl cotransport was defined as the DIOA-sensitive K influx in the presence of 30 μM bumetanide. DIOA is a [(dihydroindenyl)oxy]alkanoic acid: *R*(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl),oxy] acetic acid (Research Biochemicals International, Natick, MA). DIOA inhibits K-Cl cotransport but not Na-K-Cl cotransport (12). DIOA at 50 μM was, in our hands, a better inhibitor of K-Cl cotransport than 2 mM furosemide. These conditions, ±Cl or ±DIOA, were used to define "basal" K-Cl cotransport. Five additional criteria were used to evaluate expression of K-Cl cotransport as described in RESULTS.

In a few experiments, intracellular Mg concentration was reduced using the divalent cation ionophore A-23187, a slight modification of an earlier method (3). The concentration of A-23187 was 10 μM, and the incubation time was 20 min. The medium also contained 1 mM EDTA and $MgSO_4$ was omitted. After this preincubation, the cells were rinsed once in the Mg-free medium and the flux was measured in the same medium.

Statistics. Statistical significance of differences between means in paired experiments was calculated by Student's paired *t*-test. A value of $P < 0.05$ was taken as indicative of a significant difference.

RESULTS

Molecular cloning of the pig and human K-Cl cotransporters (KCC1) and the C. elegans K-Cl cotransporter (CE-KCC1). The cDNA for the pig homolog of the K-Cl cotransporter encodes a putative 1,086-amino acid residue protein (GenBank accession no. AF028807), which has 94% amino acid identity to the human homolog. The ATG start site in the pig sequence was confirmed by the presence of a stop codon in frame 36 bp upstream from the ATG start. The cDNA for the *C. elegans* K-Cl cotransporter (CE-KCC1) was found by sequence analysis to encode a putative 1,003-amino acid residue protein that has a calculated molecular mass of ~110 kDa (GenBank accession no. U40798 contains the genomic sequence of the clone; *C. elegans* Sequencing Consortium). This protein is shorter, by 83 amino acids, than the pig homolog, due to a shorter NH_2 terminus. CE-KCC1 is 45% similar to the human and pig KCC1 and 44% similar to the rat KCC2. Figure 1 shows the amino acid sequences of the pig and human KCC1s, CE-KCC1, and all the other known K-Cl cotransporters.

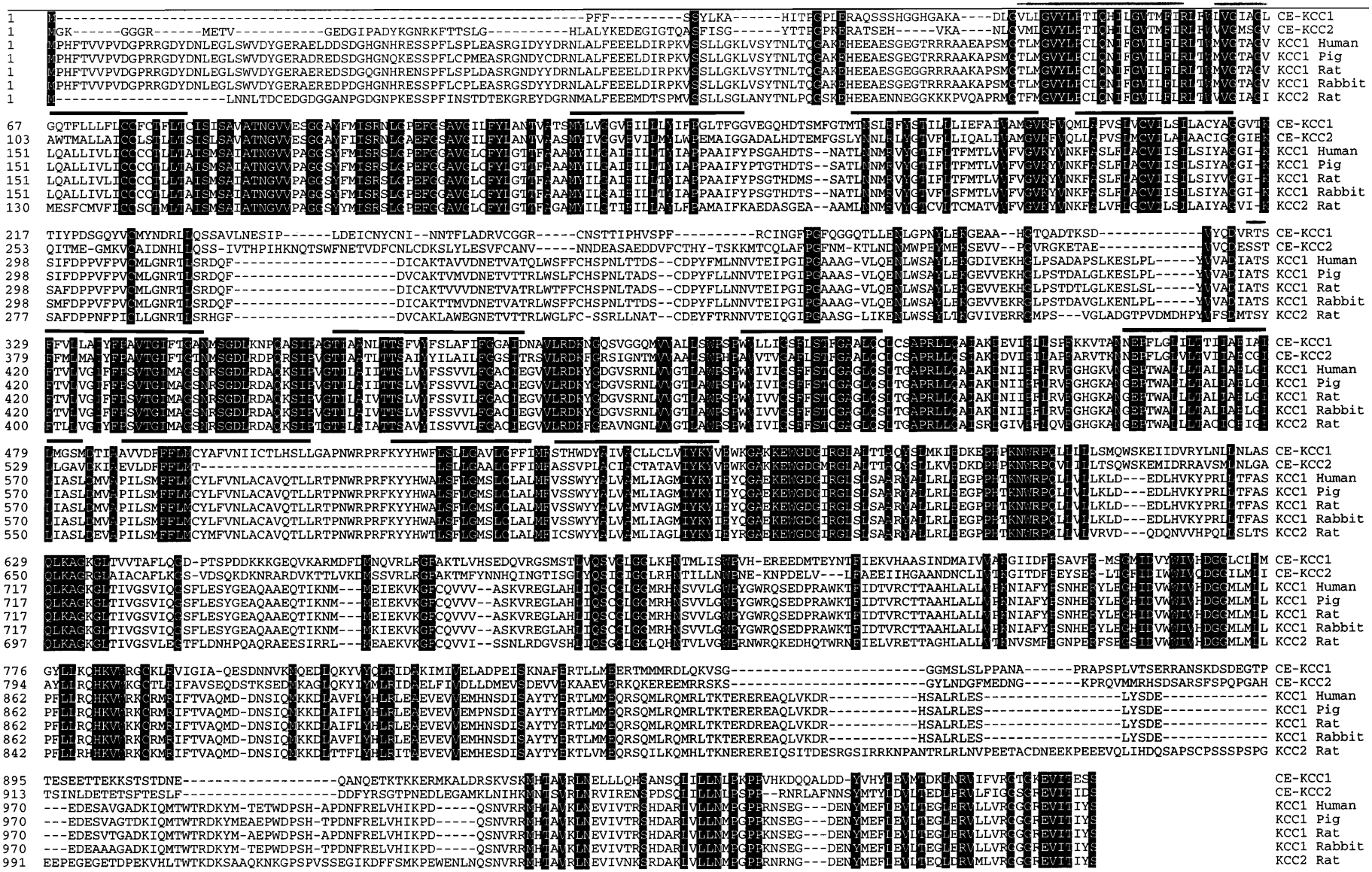

Fig. 1. Deduced amino acid sequences for all known K-Cl cotransporters: 4 mammalian KCC1s (GenBank accession nos. U55815, U55054, U55053, and AF028807 for human, pig, rat, and rabbit, respectively), rat KCC2 (GenBank accession no. U55816), and the 2 *C. elegans* homologs CE-KCC1 and CE-KCC2 (GenBank accession nos. U40798 and U23171, respectively). Amino acid residues are numbered on *left* of individual lines. Gaps have been added to obtain best alignment. Amino acids common in all cotransporters are shaded. Twelve predicted transmembrane domains are indicated for CE-KCC1 by horizontal lines. Domains 2, 6, and 9 begin in one row and conclude in the following row. Alignments were made with Lasergene (DNASTAR, Madison, WI) software using clustal method with PAM260 residue weight table. Predictions of transmembrane domains were made as described in METHODS.

Using the NCBI BLAST program, we found another *C. elegans* gene that encodes a protein of unknown function with 58% similarity to *C. elegans* CE-KCC1 cotransporter (GenBank accession no. U23171, hypothetical 112.3-kDa protein K02A2.3). We call this protein CE-KCC2. This gene, as determined by computer analysis, encodes a protein of 1,020 amino acid residues. This protein also has significant homology to the mammalian KCC cotransporters, with 42% similarity to the mammalian KCC1 cotransporters and 40% similarity to the rat KCC2. This suggests that *C. elegans* has two K-Cl cotransporter isoforms. A third cation-chloride cotransporter from *C. elegans* was reported recently (GenBank accession no. AF040650). This cotransporter shares only 23% similarity with CE-KCC1 and mammalian KCC1. It is unclear if this clone is a K-Cl cotransporter.

The pig K-Cl cotransporter clone has only 21–23% identity with the apical and basolateral Na-K-Cl cotransporters (NKCC2 and NKCC1, respectively) and with the Na-Cl cotransporter. In contrast, the Na-K-Cl and Na-Cl cotransporters are 50–60% identical (10).

These pig KCC1 and *C. elegans* CE-KCC1 proteins display significant homology to proteins from lower organisms, e.g., the open-reading frame 128 in the cyanobacterium *Synechococcus* (GenBank accession no. M18165) and the insect *M. sexta* (GenBank accession no. U17344). There is also homology to a protein in a higher plant, the tobacco plant (GenBank accession no. AF021220). A database search from the recently completed yeast genomic project has revealed only one homologous gene, YBR235w (GenBank accession no. Z36104). In *Saccharomyces cerevisiae*, this gene encodes a protein homolog of the cation-chloride cotransporter family that consists of 1,120 amino acids and is predicted to have 12 transmembrane-spanning domains and a topology similar to all of the other family members. No functions of these proteins from lower organisms had been determined until the expression of CE-KCC1 reported here.

Comparison of amino acid sequences of all the KCC cotransporters. Amino acid sequences of four mammalian KCC1s, rat KCC2, and the two *C. elegans* homologs are compared in Fig. 1. The amino acids in the KCC sequences that are identical in the same position in all of the KCCs are indicated. The twelve predicted transmembrane domains for CE-KCC1 are indicated by horizontal lines. The transmembrane domains are not quite in register among the homologs. There are substantial regions of little homology, particularly in most of the NH_2 terminus, in the COOH terminus, and in the large extracellular loop between the fifth and sixth transmembrane regions. In the rest of the sequences, there are regions of substantial identity, particularly in the first intracellular loop and in some of the transmembrane domains.

Hydropathy analysis and topology models of the pig KCC1 and C. elegans CE-KCC1. For both proteins, the Kyte-Doolittle algorithm predicts a hydrophilic NH_2-terminal domain, a very large hydrophilic COOH-terminal domain, and 12 transmembrane regions, indicated by arrows on the hydropathy plot of the pig protein in Fig. 2*A*. (The *C. elegans* protein is sufficiently similar that there seemed no reason to include it as well.) Both of the proteins contain a large hydrophilic domain between the fifth and sixth transmembrane regions, which are shown in the topology model as an extracellular loop.

Figure 2*B* shows a topology model of the pig KCC1. There are 12 putative membrane-spanning helices, extracellular loops that contain four potential N-linked glycosylation sites (Asn in positions 312, 334, 347, and 361), five intracellular loops, and the intracellular NH_2 and COOH termini. There are no consensus cAMP-dependent protein kinase phosphorylation sites. There are four consensus protein kinase C phosphorylation sites in the COOH-terminal domain (Thr in positions 748, 814, 944, and 977) and 10 consensus phosphorylation sites for casein kinase II (positions 24, 298, 734, 738, 810, 836, 942, 944, 967, and 1051).

The predicted topology of CE-KCC1 is very similar. There are 12 putative membrane-spanning helices, a large extracellular loop with three potential N-linked glycosylation sites (Asn in positions 241, 256, and 270), five intracellullar loops, a small NH_2 terminus, and a large COOH terminus. The termini of the KCCs are predicted to be intracellular on the basis of homology with NKCC (28, 36). If so, and if there are 12 transmembrane domains, the loops between membrane domains 5 and 6 are confirmed to be extracellular. There are two consensus cAMP-dependent protein kinase phosphorylation sites in positions 455 (4th intracellular loop) and 903 (intracellular COOH terminus). There are five consensus protein kinase C phosphorylation sites in the COOH-terminal domain (positions 880, 901, 919, 982, and 994). In addition, there are 11 consensus phosphorylation sites for casein kinase II (in positions 542, 648, 649, 725, 734, 893, 895, 906, 908, 919, and 994).

The topologies of both pig and *C. elegans* proteins in general resemble the other members of the cation-chloride cotransporter family, but the large extracellular domain between transmembrane domains five and six is a feature characteristic of the KCC subgroup only.

The similarities between the various K-Cl cotransporters are reflected in the phylogenetic tree in Fig. 2*C*. An early gene duplication event led to the mammalian and *C. elegans* transporters. Two subsequent duplications gave rise to the two isoforms of KCC in each group.

Tissue distribution of the human KCC1. Tissue distribution in the human was examined by Northern analysis (data not shown) and by PCR of available human libraries (Fig. 2*D*). The 3.7-kb transcript for the K-Cl cotransporter KCC1 was found in a wide variety of tissues, including heart, brain, placenta, lung, liver, muscle, kidney, and pancreas. This distribution is similar to that found by Gillen et al. (13) by Northern analysis. High levels of expression were found in the placenta, liver, and pancreas. PCR analysis of cDNA libraries demonstrated the transcript in fetal kidney,

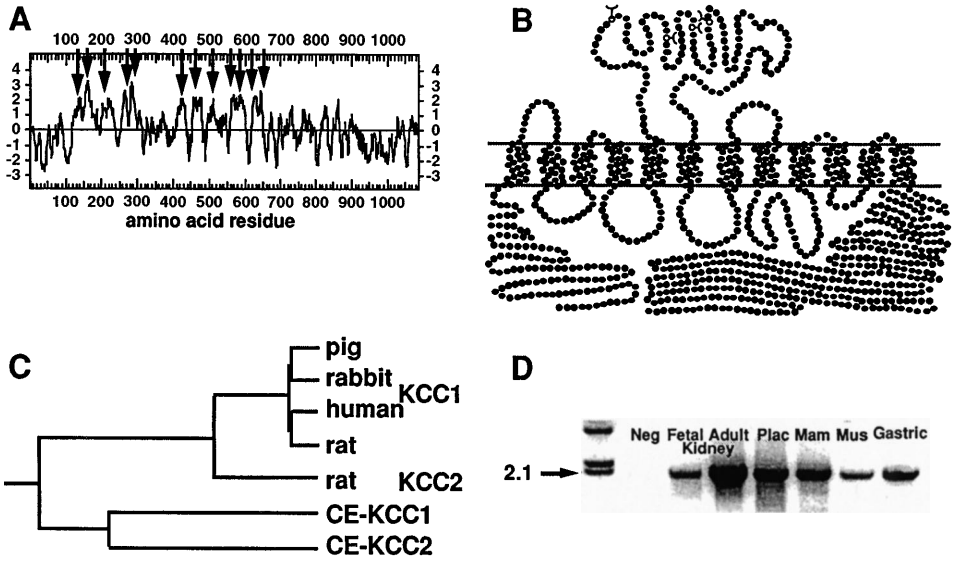

Fig. 2. *A*: Kyte and Doolittle (20) hydropathy plot of pig KCC1 cotransporter protein determined with a window of 12 amino acid residues. Algorithm predicts a hydrophilic NH_2 terminus, 12 transmembrane domains (indicated by arrows), a large loop between 5th and 6th transmembrane domain, and a huge hydrophilic COOH-terminal domain. *B*: putative topology model of pig KCC1 cotransporter protein. Solid circles represent amino acid residues. Potential N-linked glycosylation sites are open circles with branched lines indicating sugars. *C*: phylogenetic tree of K-Cl cotransporters (KCC1, ubiquitous K-Cl cotransporter; KCC2, neuronal K-Cl cotransporter; CE-KCC1 and CE-KCC2, 2 *C. elegans* homologs) (for GenBank accession nos., see legend for Fig. 1). Phylogenetic tree was constructed by unweighted pair-group method with arithmetic mean using MEGALIGN sequence analysis software (Lasergene, DNASTAR). *D*: PCR analysis done by amplification of various human cDNA libraries [Plac, placental; Mam, mammary gland; Mus, muscle; Neg, negative (blank)] with 3′ coding region of human K-Cl cotransporter gene.

mammary gland, and stomach as well. Analysis of tissue distribution of the K-Cl cotransporter, in ESTs in the GenBank database, showed the transcript in the following human tissues and cell types (in addition to the organs just listed): T-lymphocyte (EST93392, EST181920), melanocyte (ID 292021), retina (IDs 360903, 360735, 361157, 361781, 362782, and 381567), pregnant uterus (ID 486845), and 9-wk-old fetus (ID 789014). This is a wide distribution for a transporter previously thought by most workers to be confined to epithelia and red blood cells.

Organization of the human KCC1 gene. Sequencing of the 3′ region of the gene showed that the distances from the polyadenylation site of the K-Cl cotransporter to the CAAT and TATA boxes of the LCAT gene promoter are only 100 and 181 bp, respectively, downstream from the KCC1 gene (9, 13) (Fig. 3*A*).

At the 3′ end of the most downstream exon in the human gene, there is a putative 3′-mRNA processing signal (shown in Table 1) that is commonly found in other eukaryotic genes (4). An mRNA cleavage/polyadenylation signal was found to be flanked by the highly

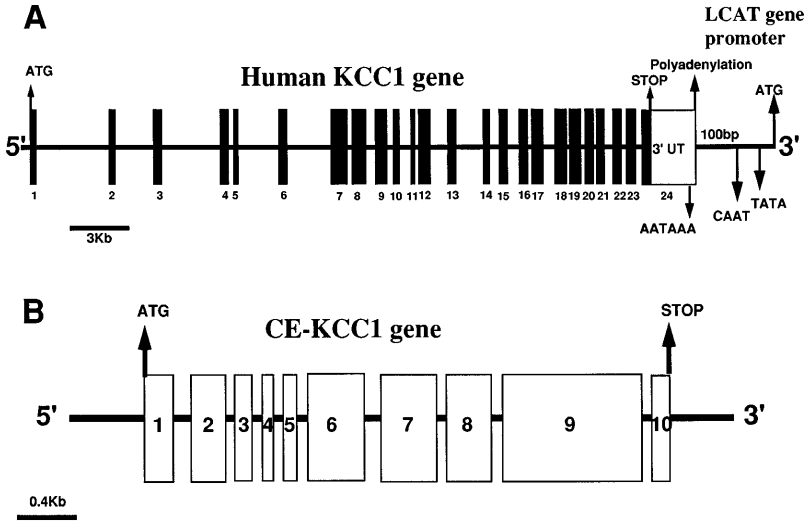

Fig. 3. Schematic gene organization maps for human K-Cl cotransporter (KCC1) and *C. elegans* cotransporter (CE-KCC1). *A*: human gene. Exons are symbolized by numbered boxes. Introns are horizontal lines between exons, with lengths of line proportional to number of base pairs. Distance between last exon of K-Cl cotransporter and TATA box of lecithin:cholesterol acyl transferase (LCAT) gene downstream is only 181 bp. Scale bar = 3 kb. Part of genomic sequence data is derived from GenBank accession no. X51966. 3′ UT, 3′ untranslated region. *B*: *C. elegans* gene. Symbols have same meanings as in *A*. Scale bar = 0.4 kb. Genomic sequence data are derived from GenBank accession no. U40798.

Table 1. *Intron-exon organization of the human KCC1 gene*

			1 ATG CCT Met Pro
	37		38
Exon 1 (115 bp)	TCG GAC G gtgagggcgc	Intron 1 (≈4.5 kb)	tgttcacag GA CAT
	Ser Asp		Glu His
	70		71
Exon 2 (95 bp)	TTT GAG gtagctgagg	Intron 2 (≈2.0 kb)	tgtttgcag GAA GAG
	Phe Glu		Glu Glu
	114		115
Exon 3 (132 bp)	GCC GAG gtgagctggtt	Intron 3 (≈3.5 kb)	ttcgcatag GCA CCC
	Ala Glu		Ala Pro
	163		164
Exon 4 (147 bp)	TGT TGT gtgagtg	Intron 4 (≈100 bp)	cccacag ACC CTG
	Cys Cys		Thr Leu
	180		181
Exon 5 (55 bp)	GTT CCA G gtcagtg	Intron 5 (≈2.5 kb)	tgctgtag CT GGG
	Val Pro A		la Gly
	225		226
Exon 6 (131 bp)	TTG CTG gtgagtcag	Intron 6 (≈2.0 kb)	tgtttcag ACC TAC
	Leu Leu		Thr Tyr
	306		307
Exon 7 (242 bp)	GTG TTT CCgtaagtaa	Intron 7 (≈150 bp)	cctcaacag G GTA TGC
	Val Phe Pr		o Val Cys
	376		377
Exon 8 (215 bp)	CTC CAG Ggtgggtcct	Intron 8 (≈300 bp)	cccctgccag AA AAC CTG
	Leu Gln G		lu Asn Leu
	432		433
Exon 9 (165 bp)	GTA ACA Ggtgagccct	Intron 9 (≈200 bp)	tttttggag GC ATC ATG
	Val Thr G		ly Ile Met
	465		466
Exon 10 (99 bp)	CTC GTG Tgtatccttt	Intron 10 (≈300 bp)	cgccgcag AC TTC AGC
	Leu Val T		yr Phe Ser
	485		486
Exon 11 (58 bp)	CGG GAC AA gtgagt	Intron 11 (≈100 bp)	ccctgcag G TAT GGC
	Arg Asp Ly		s Tyr Gly
	543		544
Exon 12 (175 bp)	CTC CGG gtgag	Intron 12 (≈400 bp)	cacag GTG TTT
	Leu Arg		Val Phe
	583		584
Exon 13 (119 bp)	TTA TCC ATgtgagctgg	Intron 13 (≈1.7 kb)	tgccgcag G TTC TTT
	Leu Ser Me		t Phe Phe
	616		617
Exon 14 (99 bp)	TAT CAC TG gtgagccat	Intron 14 (≈350 bp)	tcgtccag G GCG CTG
	Tyr His Tr		p Ala Leu
	656		657
Exon 15 (120 bp)	TAC CAA GGgtgagtga	Intron 15 (≈300 bp)	aatctgcag G GCT GAG
	Tyr Gln Gl		y Ala Glu
	691		692
Exon 16 (105 bp)	AAC TGG CGgtgagtga	Intron 16 (≈100 bp)	ccactgcag G CCG CAG
	Lys Trp Ar		g Pro Gln
	747		748
Exon 17 (169 bp)	GAG CAG gtacttgcttt	Intron 17 (≈270 bp)	tgttccccacag ACC ATC
	Glu Gln		Thr Ile
	812		813
Exon 18 (176 bp)	TTC ATT Ggtgtgt	Intron 18 (98 bp)	cctcctccccag AC ACC GTG
	Phe Ile A		sp Thr Val
	869		870
Exon 19 (169 bp)	CAT AAG gtagac	Intron 19 (88 bp)	cccttgcctcag GTC TGG
	His Lys		Val Trp
	913		914
Exon 20 (132 bp)	GAG ATG gtgagc	Intron 20 (75 bp)	acccgtccccag CAT AAC
	Glu Met		His Asn
	949		950
Exon 21 (111 bp)	CGA GAA gtcagt	Intron 21 (210 bp)	aattgtcctcag GCC CAG
	Arg Glu		Ala Gln
	1011		1012
Exon 22 (185 bp)	ATT AAG CCgtgagt	Intron 22 (148 bp)	cactacgtgtag G GAC CAA
	Ile Lys Pr		o Asp Gln
	1055		1056
Exon 23 (134 bp)	GAG AAC Tgtatcc	Intron 23 (155 bp)	tgagccctgcagAC ATG GAG
	Glu Asn T		yr Met Glu
Exon 24 (604 bp)	TTTTCAATAAAACATTGTGTAGTTCTGGGcctcctgctgccccggctctgt		

Nucleotide sequences of intron-exon junctions were determined according to strategy presented in METHODS. Exon sequences are shown in uppercase letters and intron sequences in lowercase letters. Amino acid codons bordering splice junctions are shown and numbered with 1st amino acid in K-Cl cotransport gene designated as 1. Introns and codons are randomly associated, so an intron can follow the first and second base of a codon as well as the third (e.g. Intron 5 separates G and CT of the codon specifying Ala). Exon sizes and approximate intron sizes are in parentheses. The region of mRNA cleavage/polyadenylation site in the 3′ end of exon 24 (tgctg) is underlined. A putative 3′-mRNA poly(A)$^+$ processing signal (AATAAA) in exon 24, common to many other eukaryotic genes, is also underlined. The gene organization is shown diagrammatically in Fig. 3*A*.

conserved processing sequence AATAAA about 17 bp upstream (Fig. 3*A* and Table 1).

The intron-exon organization of the human gene revealed that the KCC1 protein is encoded by 24 exons (Table 1; Fig. 3*A*). The exon lengths varied from 95 to 242 bp. All intron-exon boundaries have conventional 5′ GT and 3′ AG consensus splice sites (Table 1). Splice sites were present in regions coding for transmembrane domains as well as loops and the COOH and NH_2 termini. Introns range in length from 75 bp to 4.5 kb, and the coding region spans a total of 23 kb in the genome. Table 2 shows the primers used to define the most 5′ 17 introns.

Table 2. *Primers used to define the most 5′ 17 introns of the human KCC1 gene*

	5′ End	3′ End
Intron 1		
Forward	GCTGGATCCATGCCTCACTTCACCGTGGTGCCAGTGG	
Backward	GGAAGCCTCCAAGGGGGAAAG	
Intron 2		
Forward	GAGGCTTCCAGAGGAATTGACTACTATGACAGG	
Backward	CAGAAGAGACGATACCTTTGGGCGGATGTC	
Intron 3		
Forward	GAGCATGAGGAGGCCGAGAGT	
Backward	GCACGATGAGGAGGGCCTG	
Intron 4		
Forward	CTGCGGCTGACCTGGATGGTG	
Backward	TGGAACCACACCGTTGGTGGC	
Intron 5		
Forward	TCCATGAGTGCCATCGCCACC	
Backward	CCCCAGGATGTACATGGCTGC	
Intron 6		
Forward	GAATTTGGAGGTGCTGTGGGCCTGTGCTTCTACCTG	
Backward	GACCCCCACAAACACCACCAGGG	
Intron 7		
Forward	ACGACCGACTCCTGTGACCCCTACTTCATGCT	
Backward	CCTGGAGCACACCAGCAGCTGCCCCGG	
Intron 8		
Forward	ACGACCGACTCCTGTGACCCCTACTTCATGCT	
Backward	GAAGATGCCGACCAGCACGGT	
Intron 9		
Forward	ACGACCGACTCCTGTGACCCCTACTTCATGCT	
Backward	GTAATGATGGCCAGAATGGTCCCCACAGGG	
Intron 10		
Forward	GGGGACCTTCGTGACGCCCAG	
Backward	GTCCCGGAGAACCACACCCTC	
Intron 11		
Forward	GGGGACCTTCGTGACGCCCAG	
Backward	CGGAGGAAGGGGATGATGTTGTC	
Intron 12		
Forward	GGCGATGGTGTCAGCAGG	
Backward	GGGAGGCGATGAGGATGCCCAGCTCGGCG	
Intron 13		
Forward	CATCCCCTTCCTCCGGGTGTTTGGCCACGG	
Backward	GTGATAGTACTTGAACCG	
Intron 14		
Forward	GCAGACACTCCTGAGGACCCCCAACTG	
Backward	GTAGATCATGCCGGCGATGAG	
Intron 15		
Forward	GCGCTGTCCTTCCTGGGC	
Backward	CTTGGTGTGAGGAGGCCCCTC	
Intron 16		
Forward	GGTGACGGGATCCGAGGC	
Backward	GCCCTTGCCAGCCTTGAG	
Intron 17		
Forward	GGTGCTGCTGAAGCTGGACGAGGACCTCC	
Backward	CACCTTCTCAATTTCCATCAT	

Organization of the CE-KCC1 gene. The gene is on chromosome III, determined as described in METHODS. The genomic sequence of the CE-KCC1 gene is more compact than the human gene and spans only 3.5 kb (Fig. 3*B*). The CE-KCC1 gene has 10 exons that, on average, are bigger than the human exons, and 9 introns that are much smaller than the human ones. Table 3 shows the exon-intron organization of the CE-KCC1 gene.

Functional studies. The human, pig, and *C. elegans* K-Cl cotransporter cDNA clones were subcloned to eukaryotic expression vectors, which were used for transient and stable transfections of HEK-293 cells. The following six criteria were used to evaluate K-Cl cotransport in transfected cells: *1*) measurement of K-Cl cotransport as the bumetanide-insensitive chloride-dependent or DIOA-inhibitable K influx, called basal K-Cl cotransport; *2*) activation of cotransport by hypotonic cell swelling (7); *3*) stimulation of cotransport by *N*-ethylmaleimide (NEM) (21) (no other membrane transporter has been reported to be stimulated by NEM); *4*) stimulation of cotransport by reducing cell Mg concentration ([Mg]) using A-23187 (3) (reducing cell [Mg] apparently stimulates cotransport by reducing the concentration of Mg-ATP, the substrate for a kinase that inhibits K-Cl cotransport; Ref. 8); *5*) stimulation of cotransport by the protein kinase inhibitor staurosporine [this agent stimulates K-Cl cotransport in red blood cells (2, 5); no other membrane transporter has been reported to be stimulated by staurosporine]; and *6*) secondary activation of Na-K-Cl cotransport, probably due to cell shrinkage as a consequence of overexpression of K-Cl cotransport (C. M. Gillen and B. Forbush III, personal communication). We also tested the Cl dependence and Na independence of bumetanide-insensitive K uptake. These measurements did not provide evidence for enhanced expression of K-Cl cotransport but did help confirm that K-Cl cotransport was being measured.

In transient transfection experiments, using cells with vector only as controls, the human, pig, and *C. elegans* constructs were evaluated for expression of K-Cl cotransport by three criteria: *1*) basal K-Cl cotransport, *2*) stimulation of K-Cl cotransport by NEM, and *3*) secondary activation of Na-K-Cl cotransport. Results on control and transiently transfected cells are presented in Fig. 4. Increased expression of K-Cl cotransport was demonstrated clearly in all three clones by the criteria of stimulation by NEM and secondary stimulation of Na-K-Cl cotransport. By the criterion of increased basal K-Cl cotransport, the increased expression in the transfected cells was modest at best (and not statistically significant) with the human and pig constructs, and not evident with *C. elegans*. Examples are shown below of increased expression of basal K-Cl cotransport. In all experiments, basal cotransport was the most problematic criterion. We discuss below a likely explanation for the low level of expression when measured as basal cotransport.

Figure 5 shows enhanced K-Cl cotransport evaluated by six criteria in clones of cells stably transfected with the human construct for the K-Cl cotransporter. Re-

Table 3. *Intron-exon organization of the C. elegans CE-KCC1 gene*

	79		1 ggaattcggctt ATG CCA Met Pro
Exon 1 (237 bp)	TTC TGT gtaagt Phe Cys	Intron 1 (91 bp)	80 attaatttacag ACT TTC Thr Phe
	159		160
Exon 2 (241 bp)	GAT ACC Tgtttgt Asp Thr S	Intron 2 (46 bp)	tccaaattacag CC ATG TTC er Met Phe
	196		197
Exon 3 (110 bp)	GCA CCG gtaaga Ala Pro	Intron 3 (50 bp)	gagtatttttagGTT TCA Val Ser
	225		226
Exon 4 (86 bp)	GGA CAA TAgtgagg Gly Gln Ty	Intron 4 (49 bp)	tgtggttttcag T GTT TGT r Val Cys
	256		257
Exon 5 (93 bp)	AAT ATA AAgtgagc Asn Ile As	Intron 5 (46 bp)	atattgtttcag C AAC ACT n Asn Thr
	394		395
Exon 6 (414 bp)	CGA GAC AAgtactt Arg Asp Ly	Intron 6 (52 bp)	catatctttcag A AAT GGG s Asn Gln
	525		526
Exon 7 (393 bp)	TAC CAT TGgtatgt Tyr His Tr	Intron 7 (54 bp)	atatttttacag G TTC CTG p Phe Leu
	644		645
Exon 8 (358 bp)	TTG CAG gtttgt Leu Gln	Intron 8 (58 bp)	tttactttcagGGA GAT Gly Asp
	967		968
Exon 9 (970 bp)	AAA GAT Cgtaagt Lys Asp G	Intron 9 (49 bp)	aaaattttccag AG CAA GCA ln Gln Ala
	1003		
Exon 10 (107 bp)	AGC TCG TAActtcacaaagtacgcttgttatcaagaaaatatacatatctatggtttt Ser Ser *		

Nucleotide sequences of intron-exon junctions were determined according to strategy described in METHODS and by comparison of genomic sequence of CE-KCC1 gene (GenBank accession number U40798) (35) and cDNA sequence. Exon sequences shown in uppercase letters and intron sequences in lowercase letters. Amino acid codons bordering splice junctions are numbered with ATG initiation codon designated 1. Exon sizes and approximate intron sizes are given in parentheses. The asterisk in exon 10 indicates the stop codon. Gene organization is shown diagrammatically in Fig. 3*B*.

sults of three experiments are shown. In Fig. 5*A*, enhanced expression was evaluated by four criteria: *1*) basal cotransport, *2*) stimulation by NEM, *3*) stimulation by hypotonic swelling, and *4*) secondary stimulation of Na-K-Cl cotransport. In Fig. 5*B*, expression was evaluated by two criteria: *1*) basal cotransport and *2*) stimulation by staurosporine. In Fig. 5*C*, results are shown for three clones of cells; one criterion, basal cotransport, was employed to test for enhanced expression of the cotransporter. This experiment was carried out in Na-free medium.

In Fig. 5*A*, there was again no increased expression of basal K-Cl, but there was increased expression of cotransport in the clone by the criterion of secondary stimulation of Na-K-Cl cotransport. Further evidence for increased expression was the increased stimulation by NEM and the threefold stimulation by hyposmotic cell swelling.

In the experiment shown in Fig. 5*B*, there were two criteria for enhanced expression: *1*) basal cotransport and *2*) stimulation by staurosporine. Basal cotransport was enhanced nearly twofold in the clone, although the statistical significance of the increase was marginal. Staurosporine greatly stimulated cotransport in the control cells, and the transport was higher by twofold in the clone pretreated with staurosporine. In red blood cells, staurosporine stimulates K-Cl cotransport to a greater extent than does swelling or NEM (5, 8, 21).

Figure 5*C* shows an experiment with three clones in Na-free medium. There was significantly enhanced basal cotransport in all three clones. The K fluxes did not require Na in the medium and therefore were not mediated by Na-K-Cl cotransport. There was significant stimulation in clone 7/10, the same clone in which there was marginal or no stimulation in Figs. 5*A* and 5*B*. There is no obvious explanation for this variability; speculation is possible but not likely to be fruitful.

The effect of reduced cell [Mg], a criterion employed in the experiments on the *C. elegans* cotransporter, was not tested on the human cotransporter. It was judged that five criteria were sufficient. When positive results were not obtained with staurosporine in the *C. elegans* experiments, an additional criterion, low cell [Mg], was added to those experiments.

Figure 6 shows results of experiments on a clone of cells stably transfected with the *C. elegans* construct. Figure 6*A* shows tests for enhanced cotransport in the clone by two criteria: *1*) enhanced basal cotransport and *2*) enhanced secondary stimulation of Na-K-Cl cotransport.

In the experiments in Fig. 6*B*, the effect of reducing cell [Mg] was tested. Cotransport was stimulated by

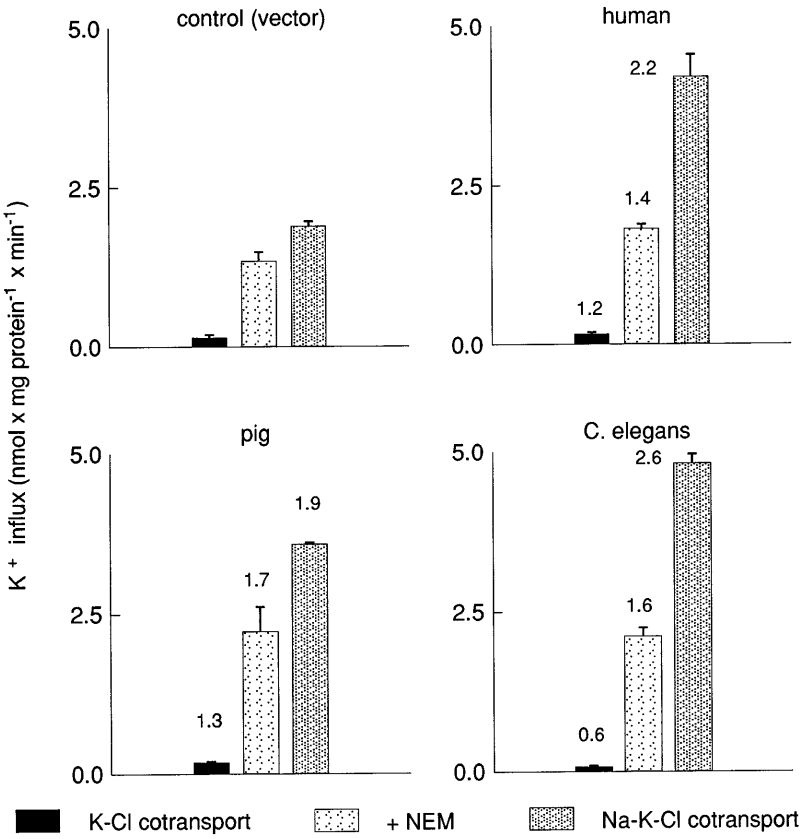

Fig. 4. K-Cl cotransport in HEK-293 transiently transfected with human, pig, and *C. elegans* K-Cl cotransporters. Control cells were transfected with vector alone. Treatment of cells and measurement of fluxes were carried out as described in METHODS. Fluxes (in nmol·mg protein^{-1}·min^{-1}) are shown for Na-K-Cl cotransport (±50 μM bumetanide), K-Cl cotransport (10 μM bumetanide ± Cl, with gluconate as substitute anion), and stimulation of K-Cl cotransport with *N*-ethylmaleimide (NEM) (1 mM). Fractional increases for each condition in transfected cells compared with controls are indicated by numbers at top of bars. In transfected cells, no increases in basal cotransport were statistically significant. For increased stimulation by NEM, $P < 0.03$, 0.05, and 0.025 for human, pig, and *C elegans*, respectively. For increased stimulation of Na-K-Cl cotransport, $P < 0.01$, 0.005, and 0.005 for human, pig, and *C. elegans*, respectively. Similar results were obtained in 4 other experiments of same design.

reduced cell Mg in control cells and was stimulated further in the clone. Basal cotransport was enhanced nearly twofold, but this increase was not statistically significant.

Figure 6*C* shows the results of an experiment with transport measured in Na-free medium. Enhanced expression was evaluated by three criteria: *1*) basal cotransport, *2*) stimulation by NEM pretreatment, and *3*) stimulation by hypotonic swelling. Under all of these conditions, there was significantly enhanced expression of K-Cl cotransport in the clone. That the enhancement occurred in Na-free medium is confirmation that the increased K influx is mediated by the K-Cl cotransporter.

Figure 6, *A–C*, shows enhanced expression of the *C. elegans* K-Cl cotransporter stably expressed in a human cell line. There was enhanced expression of the cotransporter as judged by five criteria. Staurosporine was also tested in several experiments on the *C. elegans* cotransporter. A consistently significant increase in cotransport was not found. This could have been due to experimental problems of unknown nature. It is not likely to mean a difference in the signal transduction pathway regulating the *C. elegans* cotransporter because only the cotransporter protein is transfected into the cells. The enzymes controlling the cotransporter must be endogenous enzymes of the HEK cells.

K-Cl cotransport in cells transfected with the mammalian constructs was defined in most experiments of the Cl-dependent, bumetanide-insensitive K influx. In cells expressing the *C. elegans* construct, Cl dependence proved to be a less reliable measure of K-Cl cotransport than with the mammalian constructs. Therefore, DIOA sensitivity was used to define K-Cl cotransport. This agent inhibits K-Cl cotransport but not Na-K-Cl cotransport (12). DIOA did provide consistent measures of cotransport by the *C. elegans* cotransporter. The explanation for these more consistent results with DIOA is unclear. It was confirmed that DIOA inhibited the mammalian cotransporters and that it did not inhibit Na-K-Cl cotransport. In our hands, 50 μM DIOA gave maximal inhibition of K-Cl cotransport, both of endogenous cotransport and cotransport in the clones. Payne (25) reported that 100 μM DIOA inhibited only ~60% of cotransport by rat KCC2, which was inhibited by 2 mM furosemide. The difference in sensitivities may be due to the difference in structure between the two proteins.

A number of experiments were carried out on the steady-state kinetics of K-Cl in control cells and in cells stably transfected with CE-KCC1 in the hope of determining if there is a difference in the $K_{1/2}$ for K between CE-KCC1 and the endogenous cotransporter of HEK cells. There were two difficulties with these experiments. First, owing to the low level of enhanced expression in the transfected cells, the rates of K transport through the endogenous cotransporter and through CE-KCC1 are roughly equal at physiological K. Therefore, only large differences in $K_{1/2}$ could be detected. Second, the $K_{1/2}$ for mammalian KCC1 is quite high (apparently well in excess of 25 mM) (13).

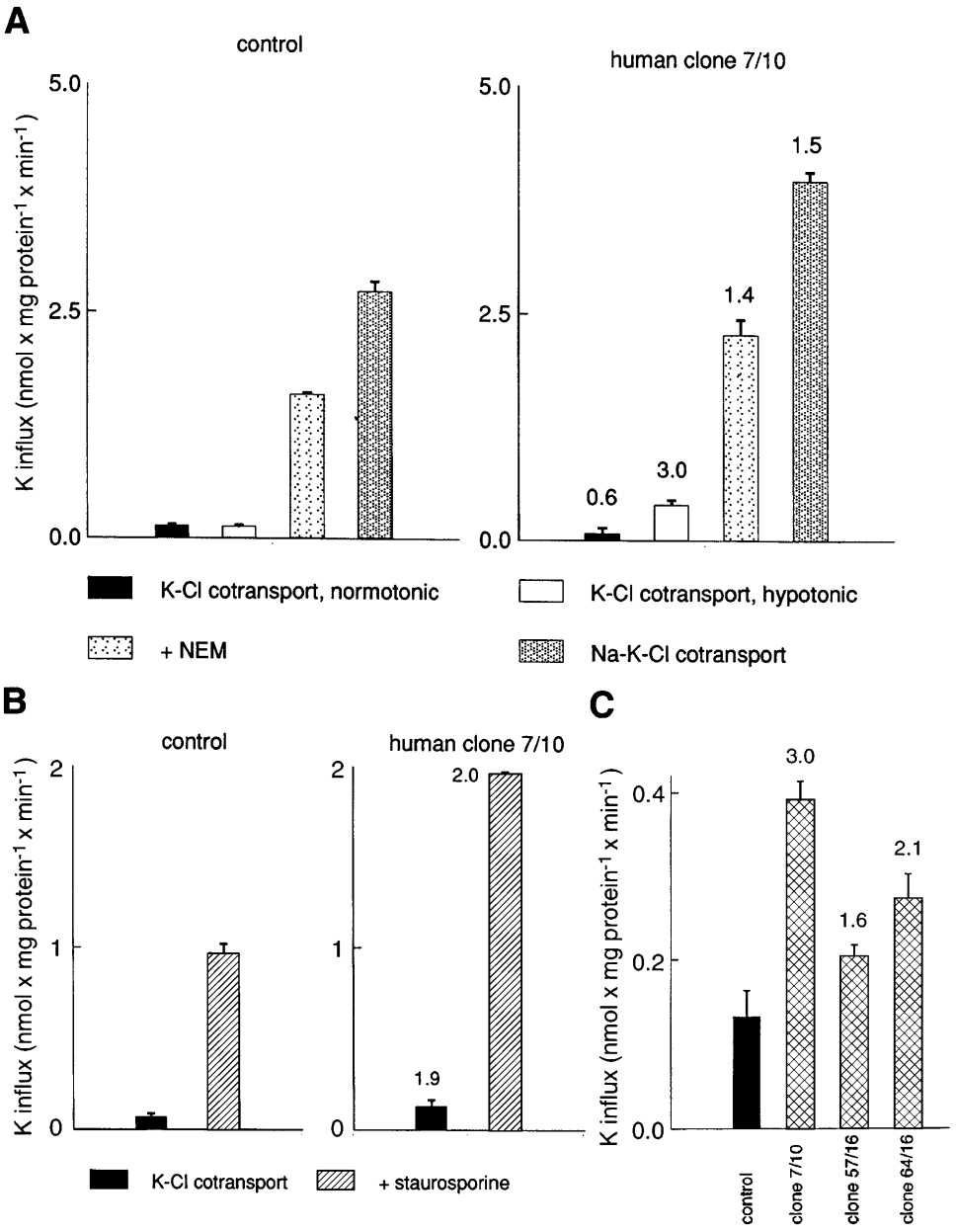

Fig. 5. K-Cl cotransport into control HEK-293 cells and clones of cells stably transfected with human K-Cl cotransporter. K-Cl cotransport was defined as Cl-dependent K influx in presence of 10 μM bumetanide. Treatment of cells and measurement of fluxes were carried out as described in METHODS. Fractional increases for each condition in transfected cells compared with controls are indicated by numbers at *top* of bars. *A*: criteria for enhanced expression were basal cotransport, stimulation of cotransport by hypotonic swelling (hypotonic medium, 150 mosmol/kgH_2O), stimulation by NEM pretreatment (1 mM), and secondary stimulation of Na-K-Cl cotransport. In the clone, basal cotransport was not increased. For increases in the clone in stimulation by swelling, by NEM, and in Na-K-Cl cotransport, $P < 0.025$, 0.025, and 0.005, respectively. Similar results were obtained in 2 other experiments of the same design. *B*: expression of K-Cl cotransport evaluated as basal cotransport and as staurosporine-stimulated cotransport (staurosporine pretreatment: 2.5 μM, 10 min). Increase in basal cotransport in clone was not quite significant ($0.05 < P < 0.06$). For increase in stimulation by staurosporine in the clone, $P < 0.005$. Similar results were obtained in 4 other experiments of similar design. *C*: K-Cl cotransport measured in Na-free medium in control cells and in 3 clones of cells stably transfected with the human K-Cl cotransporter. Na substitute was *N*-methyl-D-glucamine. K-Cl cotransport was defined as bumetanide-insensitive, furosemide-inhibitable K influx; furosemide concentration was 2 mM. For increases in basal cotransport in clones 7/10, 57/16, and 64/16, $P < 0.01$, 0.05, and 0.025, respectively.

In six experiments, the mean $K_{1/2}$ for the endogenous cotransporter was ~50 mM (results not shown). In transfected cells, which transport K through both the endogenous and the *C. elegans* cotransporters, $K_{1/2}$ was not distinguishable from 50 mM. The tentative conclusion is that the $K_{1/2}$ for K of CE-KCC1 does not differ greatly from that of mammalian KCC1 despite the substantial differences in structure of the two proteins. The difference is certainly not as great as that between rat KCC2 ($K_{1/2}$ 6.6 mM; Ref. 25) and endogenous KCC1 in HEK cells.

DISCUSSION

We report here the cloning, sequencing, and functional expression of the human, pig, and *C. elegans* K-Cl cotransporters. We present the organization of the human and *C. elegans* genes as determined from data in the GenBank and from long PCR studies.

Each of the proteins in the cation-chloride cotransporter family has a central, relatively conserved hydrophobic domain that is predicted to contain 12 transmembrane domains. Unlike the other family members, which have the largest extracellular loop between the seventh and eighth membrane domains (10, 11, 28, 36), the largest extracellular loop of the mammalian KCC1 and CE-KCC1 isoforms of the K-Cl cotransporter is between the fifth and sixth membrane-spanning domains. Otherwise, KCC1 and CE-KCC1 have topological features in common with other members of the family, including an NH_2 terminus that is the least conserved region of the protein and a moderately conserved large COOH-terminal domain (24). The fact

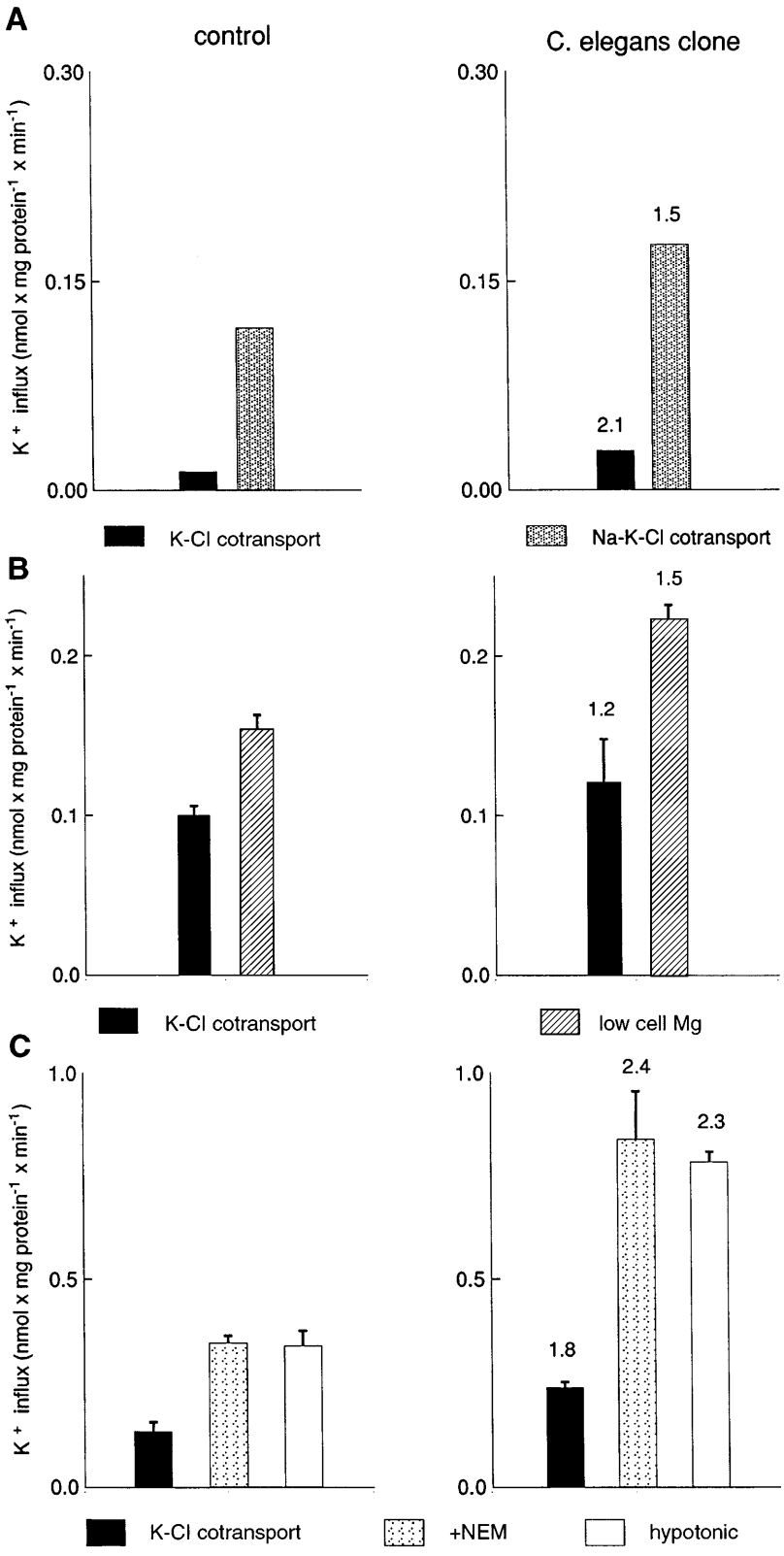

Fig. 6. K-Cl cotransport into control cells and into a clone of cells stably transfected with *C. elegans* cotransporter. K-Cl cotransport is defined as DIOA-inhibitable K influx in presence of 30 μM bumetanide. Treatment of cells and measurement of fluxes were carried out as described in METHODS. Fractional increases for each condition in transfected cells compared with controls are indicated by numbers at *top* of bars. *A*: 2 criteria for enhanced expression of cotransport in clone were employed: basal cotransport and secondary stimulation of Na-K-Cl cotransport. These fluxes are from slopes of uptake curves, measured from 0.5 to 5 min. Correlation coefficients of slopes (calculated by method of least squares) were, in order of fluxes from *left* to *right*, 0.995, 0.995, 0.914, and 0.995. Similar results were obtained in 6 other experiments of similar design. *B*: expression of cotransport evaluated as basal cotransport and as cotransport stimulated by reducing cell [Mg] by pretreatment of cells with divalent cation ionophore A-23187 (10 μM, 20 min) (3). In the clone, increase in basal was not statistically significant. For increase in cotransport caused by low [Mg] in clone, $P < 0.01$. Similar results were obtained in 3 other experiments of similar design. *C*: K-Cl cotransport in Na-free medium; *N*-methyl-D-glucamine was substitute cation. Three criteria were employed to test for increased expression of cotransporter in the clone: basal cotransport, stimulation by NEM (1 mM), and stimulation by hypotonic swelling (hypotonic medium: 150 mosmol/kgH_2O). For increases in the clone in basal cotransport, and cotransport stimulated by NEM and by swelling, $P < 0.025$, 0.025, and 0.005, respectively. Criteria for enhanced K-Cl cotransport were tested with similar results in other experiments carried out in Na-containing medium: basal cotransport, 6 experiments; swelling, 5 experiments; NEM, 5 experiments; and staurosporine, 4 experiments.

that the NH_2 terminus of the *C. elegans* protein is shorter than the human and pig cotransporters by 82 amino acids suggests that at least part of the mammalian K-Cl cotransporter NH_2 terminus is not important to the transport function of the protein because the functions of the human and *C. elegans* transporters are very similar. Alignment of the full-length proteins of the entire family shows that the loop between putative spanners 2 and 3 and a portion of spanner 6 toward the intracellular membrane surface are conserved in all

members of the family (24) and may be related to functional features shared by all family members, such as chloride binding and translocation of substrate ions across the membrane. Knowledge of the structure of the K-Cl cotransporters can guide further studies using mutagenesis and construction of chimeric proteins (e.g., K-Cl/Na-Cl cotransporter) to find structural domains that are important for the binding and transport of the substrate ions and to study, at the molecular level, the role of the transporters in the regulation of cell volume control.

The gene organization of the human KCC1 gene is similar to that of the other members of the family that have been determined, mouse NKCC1 (30) and human NKCC2 and NCC, in which mutations were described as the causes for Bartter's and Gitelman's syndromes (33, 34). All the genes in the family have 24–27 exons with an average of 51 amino acids coded per exon. The human genes for NKCC2 and NCC both have 26 relatively small exons, and their intron/exon boundaries are more similar to each other than to the intron/exon boundaries in KCC1. The human *KCC1* gene is the smallest in the family, spanning 23 kb. The human NCC and the mouse NKCC1 genes span 55 kb and 75 kb, respectively. The human NKCC2 gene is the longest, spanning ~80 kb. Knowing the gene organization of the human KCC1 could help in the search for diseases that can be related to K-Cl cotransport in the same way that Bartter's syndrome was shown to be caused, in part, by a mutation in the NKCC2 cotransporter (33).

Members of the cation-chloride cotransporter family are known to have a number of splice variants (26). The organization of the K-Cl cotransporter gene could also help in identifying splice variants specific for particular tissues (E. Holtzman, unpublished observations) or identifying splice variants specific in cotransporter proteins in the apical and/or basolateral membranes in epithelial cells. Pellegrino et al. (29) have detected two mRNA isoforms of human erythroid-KCC1 that resulted in COOH-terminal truncated proteins (73 amino acids and 17 amino acids, respectively). The first isoform is a splice variant that lacks the regions encoded by exon 23 and parts of exon 24.

The LCAT gene begins very near the 3′ end of the KCC1 gene. LCAT deficiency is well characterized molecularly, but a literature search uncovered no reports of large deletions in the KCC1-LCAT region. Such a deletion could reveal the phenotype of a deletion in the KCC1 gene (18).

There is evidence that K-Cl cotransport activity is involved in the process of sickling of red blood cells in sickle cell disease (1, 17). The relationship of hemoglobin S (HbS) to K-Cl cotransport activity has not been established at the molecular level (15). One possibility is that a polymorphism in the K-Cl cotransporter gene is prevalent in the affected population and results in elevated rate of transport and enhanced severity of the disease in those individuals with both the HbS mutation and the polymorphism of the cotransporter gene. Knowledge of the structure of the KCC1 gene may help determine if there is such an association.

Gillen et al. (13) cloned and sequenced the K-Cl cotransporter from rat, rabbit, and human and studied the expression of the rabbit cotransporter using c-myc-tagged KCC1. We have cloned the cotransporter from human, pig, and *C. elegans* and have mainly studied the expression of the human and *C. elegans* cotransporters. We showed enhanced expression of the cotransporter in both transiently and stably transfected cells. The absolute K-Cl cotransport fluxes and the extent of enhanced expression in the transfected cells were similar in the present study and in that of Gillen et al. (13). The observed expression in transiently transfected cells should permit more rapid assay of mutated and chimeric genes than is possible with stable transfections.

As discussed, we employed a total of six criteria in different experiments to determine whether expression of K-Cl cotransport was enhanced in cells stably transfected with the human or *C. elegans* clone. Five criteria were used for the human cotransporter and five for *C. elegans*; four of the criteria were used for both species. By all criteria used, enhanced K-Cl cotransport was demonstrated in cells stably transfected with DNA for the cotransporters from both species. The increased expression was modest, but the employment of multiple criteria helped to make a convincing case that the human and *C. elegans* genes we have isolated indeed code for the K-Cl cotransporter. The pig construct was shown by two criteria to enhance K-Cl cotransport in transiently transfected cells.

Gillen et al. (13) characterized the function of rabbit and human KCC1 stably transfected in HEK-293 cells. In that study, enhanced expression of K-Cl cotransport was evaluated by increased furosemide-inhibitable K efflux, swelling-activated furosemide-inhibitable K efflux, and stimulation of furosemide-sensitive K influx by NEM. Na independence and Cl dependence of K influx in NEM-treated cells confirmed that the K flux was K-Cl cotransport. We showed Cl dependence and Na independence in cells under more physiological conditions, i.e., not treated with NEM. We utilized three additional criteria, stimulation by staurosporine (human clone) and by reduced cell [Mg] (*C. elegans* clone), and secondary stimulation of Na-K-Cl cotransport (both clones).

Activation of K-Cl cotransport can cause cell shrinkage due to the osmotically obliged efflux of water (15). Overexpression of K-Cl cotransport will cause the same change more rapidly and/or to a greater extent. This, in turn, will reduce the rate of K-Cl cotransport by shrinkage inactivation and will cause shrinkage activation of Na-K-Cl. A steady state will result at a reduced cell volume, maintained by a slightly elevated rate of K-Cl cotransport in slightly shrunken cells due to the overexpression of K-Cl cotransport. (If the steady state were not at a reduced volume, Na-K-Cl cotransport would not remain elevated.) This would explain why increased expression of K-Cl cotransport was often low when evaluated as the basal rate of cotransport. When

evaluated by other criteria, such as stimulation by swelling or NEM, the increased expression of K-Cl cotransport was greater than when evaluated as basal cotransport.

We consistently found this association between stimulation of Na-K-Cl cotransport and enhanced K-Cl cotransport. In epithelial cells, there is coordination of rates of transport across the apical and basolateral membranes. This coordination, called crosstalk, is not thoroughly understood (32). The association between Na-K-Cl and K-Cl cotransport seen here resembles such a crosstalk mechanism, although it may not involve an apical Na-K-Cl cotransporter and a basolateral K-Cl cotransporter as it would in epithelia.

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