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A Novel N-terminal Splice Variant of the Rat H^+-K^+-ATPase α2 Subunit

CLONING, FUNCTIONAL EXPRESSION, AND RENAL ADAPTIVE RESPONSE TO CHRONIC HYPOKALEMIA*

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The H^+-K^+-ATPase of renal collecting duct mediates K^+ conservation during chronic hypokalemia. K^+ deprivation promotes H^+-K^+-ATPase α2 (HKα2) gene expression in the medullary collecting duct, the principal site of active K^+ reabsorption, suggesting that this isozyme contributes to renal K^+ reclamation. We report here that alternative transcriptional initiation and mRNA splicing give rise to distinct N-terminal variants of the HKα2 subunit. Sequence analysis and *in vitro* translation revealed that HKα2a corresponds to the known HKα2 cDNA (Crowson, M. S., and Shull, G. E. (1992) *J. Biol. Chem.* 267, 13740–13748), whereas HKα2b represents a novel variant truncated by 108 amino acids at its N terminus. HKα2b mRNA contains a complex 5′-untranslated region with eight upstream open reading frames, features implicated in translational regulation of other genes. Heterologous expression of HKα2b with and without the gastric H^+-K^+-ATPase β subunit in HEK 293 cells indicated that this variant encodes a K^+ uptake mechanism that is relatively Sch 28080-resistant, partially sensitive to ouabain, and appears to require coexpression with the gastric H^+-K^+-ATPase β subunit for optimal functional activity. Northern analysis demonstrated that both subtypes (HKα2b > HKα2a) are expressed abundantly in distal colon and modestly in proximal colon and kidney. Moreover, the abundance of the two mRNAs increases coordinately among the renal zones, but not in colon, with chronic K^+ deprivation. These results demonstrate the potential for complex control of HKα2 gene expression by transcriptional and posttranscriptional mechanisms not recognized in other members of the Na^+-K^+-ATPase/H^+-K^+-ATPase family.

The maintenance of body potassium (K^+) balance is critical to the normal function of all cells. Perturbations in K^+ homeostasis disrupt normal cell growth and division, metabolism, volume and osmotic regulation, acid-base economy, and the excitability of nerve and contractile cells. The kidney is the principal arbiter of body K^+ balance in mammals, adjusting K^+ excretion to match large variations in dietary K^+ intake. The late distal tubule and collecting duct have the dual ability to secrete and reabsorb K^+ as needed to effect this balance (1, 2). In response to chronic dietary K^+ deprivation, these segments, in particular the OMCD[1] actively reclaim filtered K^+. Physiological, biochemical, and molecular biological studies have shown that this adaptation is principally attributable to increased expression and/or activity of an H^+-K^+-ATPase in the luminal membrane of these segments (2–9). A similar transport system(s) has been identified in the apical membrane of mammalian distal colon, where it, too, effects active K^+ absorption (10, 11). Although active K^+ absorption in the distal colon is enhanced during K^+ depletion (11) and participates to a limited degree in restoring K^+ balance, the identity of the specific K^+-ATPase that is up-regulated remains controversial (12).

The H^+-K^+-ATPases constitute a subfamily of isozymes that belong to the X^+-K^+-ATPase multigene family, which also includes the Na^+-K^+-ATPase isoforms. The X^+-K^+-ATPases share common catalytic and ion transport mechanisms and an apparent requirement for heterodimeric (α:β) structure. The X^+-K^+-ATPase α subunits exhibit considerable (~65%) structural homology and contribute most of the functional properties of the holoenzymes, but they can be distinguished to a degree from one another on the basis of organ distributions and sensitivities to the inhibitors ouabain and Sch 28080 (13). To date, three distinct H^+-K^+-ATPase α subunits have been cloned from mammals, structurally characterized, and expressed in heterologous systems. The H^+-K^+-ATPase α1 subunit (HKα1) was first cloned from and is principally expressed in stomach (14), where it plays a major role in gastric acid secretion. Messenger RNA encoding this gene was also identified in the renal collecting duct (3). The pharmacological signature of the HKα1 protein is its high sensitivity to inhibition by Sch 28080 and its complete resistance to inhibition by ouabain (15, 16). The H^+-K^+-ATPase α2 cDNA was first cloned from rat distal colon (17), where it is abundantly expressed, and lower levels of HKα2 mRNA were reported in proximal colon (17), uterus (17), and kidney (5–8). Expression of the HKα2 subunit with the known rat X^+-K^+-ATPase β subunits (16) or toad bladder H^+-K^+-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U94911–U94913.

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[1] The abbreviations used are: HKα2, H^+-K^+-ATPase α2 subunit, also termed the colonic H^+-K^+-ATPase α subunit; HKα1, H^+-K^+-ATPase α1 subunit, also termed the gastric H^+-K^+-ATPase α subunit; HKβ_g, gastric H^+-K^+-ATPase β subunit; OMCD, outer medullary collecting duct; RACE, rapid amplification of cDNA ends; C/EBP, CCAAT enhancer binding protein; NF-κB, nuclear factor kappa B; HNF-4, hepatocyte nuclear factor-4; IRF-1, interferon regulatory factor-1; GR, glucocorticoid receptor; UTR, untranslated region; u, upstream; ORF, open reading frame; Sch 28080, 2-methyl,8-(phenylmethoxy)imidazo(1,2-*a*)pyridine 3-acetonitrile; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ATPase β subunit (18) in *Xenopus laevis* oocytes resulted in the appearance of active H^+-K^+ exchange that was virtually resistant to Sch 28080 and partially inhibited by ouabain. When HKα2 was expressed without an exogenous β subunit in Sf9 cells, the resultant K^+-ATPase activity was Sch 28080- and ouabain-resistant (19). A third H^+-K^+-ATPase α subunit cDNA, termed ATP1AL1 (or H^+-K^+-ATPase α4), was cloned from a human skin cDNA library (20), and transcripts encoding this gene product were also detected in human brain and kidney but not colon (20). Coexpression of the ATP1AL1 subunit and the rabbit gastric H^+-K^+-ATPase β subunit ($HK\beta_g$) in *Xenopus* oocytes (21) or HEK 293 cells (22) resulted in the expression of functional H^+-K^+ pumps that were partially sensitive to both Sch 28080 and ouabain.

Recent studies by our laboratory and others have shown that chronic K^+ deprivation enhances HKα2, but not HKα1 (3), gene expression in the OMCD (5–8) and proximal portion of the inner medullary collecting duct (6) of rats. In one of these studies (8), HKα2 protein levels, but not mRNA levels, were enhanced in the outer medulla of K^+-deprived rats, suggesting the potential operation of translational or post-translational control mechanisms. In contrast to kidney, chronic hypokalemia does not appear to alter HKα2 mRNA (5, 8) or protein (8) abundance in rat distal colon. Moreover, recent work demonstrating disparate effects of adrenalectomy, dexamethasone treatment (5), and dietary Na^+ depletion (8) on HKα2 abundance in the rat outer medulla and distal colon indicated that cell type-specific regulatory mechanisms govern HKα2 gene expression in these tissues.

Since both transcriptional and translational control mechanisms, as well as alternative mRNA splicing, can lead to regulated, tissue-specific gene expression, we hypothesized that these mechanisms might operate to confer structural and/or regulatory diversity to the HKα2 subunit gene. Although the structural organization of the rat and human HKα1 (23) and human *ATP1AL1* (24) genes is known, that of the rat HKα2 gene has not been described. We report here that distinct transcription initiation sites in the rat HKα2 gene and alternative mRNA splicing, combined regulatory mechanisms not known to be utilized by other members of the X^+-K^+-ATPase α subunit family, direct the synthesis of two N-terminal HKα2 variants that are expressed principally, if not exclusively, in the kidney and colon and that appear to respond coordinately in kidney to chronic K^+ deprivation.

EXPERIMENTAL PROCEDURES

Animal Protocols—Male Sprague-Dawley rats (180–220 g) were fed normal rat chow (150 mEq KCl/kg chow, TD88081, Harlan Teklad) or a nominally K^+-free (TD88082, Harlan Teklad) diet for 2 weeks. This K^+-restriction protocol reproducibly results in significant hypokalemia (3) and has been used in our previous studies (3, 6).

Oligonucleotide Primers—PCR primers not included in specific kits were synthesized by Genosys, Inc. (The Woodlands, TX). The sequences of the various HKα2 subunit primers are presented in Fig. 1*A*, and those of the $HK\beta_g$ subunit are given below.

5′-RACE and Cloning of H^+-K^+-ATPase α2b cDNA—The 5′-RACE protocol was performed using the Marathon™ cDNA Amplification Kit (CLONTECH, Palo Alto, CA), according to the manufacturer's instructions. First strand cDNAs were generated from 1 μg of rat kidney poly(A)$^+$ RNA, using Moloney murine leukemia virus reverse transcriptase and a modified locking oligo(dT) primer containing two degenerate nucleotide positions at its 3′ end provided with the kit. Second strand synthesis was accomplished with a mixture of *Escherichia coli* DNA polymerase I, RNase H, and *E. coli* DNA ligase. After creation of blunt ends with T4 DNA polymerase, the double-stranded cDNA was ligated to adapter primer 1 furnished with the kit, using T4 DNA ligase. The anchor-ligated cDNAs were then subjected to 5′-RACE using a nested primer (adapter primer 2, supplied with the RACE kit) complementary to adapter primer 1, HKα2-specific reverse primer (P1, Fig. 1*A*) complementary to nucleotides +344 to +325 of the published HKα2 cDNA sequence (17), and the components of the Advantage™ cDNA Amplification Kit (CLONTECH). PCR cycling conditions were as follows: 94 °C × 1 min, followed by 28 cycles of 94 °C × 30 s, 68 °C × 4 min, and a final step of 68 °C × 4 min. Ten μl of the amplified products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining and UV shadowing. The final amplicons were then subcloned into the plasmid vector pCR2.1™ (Invitrogen) and sequenced on both strands by a cycle sequencing method.

To establish the coding sequences 3′ to the alternative splice site of the HKα2b variant (see "Results"), the complete encoding DNA was PCR-amplified from oligo$(dT)_{17}$-primed rat kidney cDNA, using the HKα2b-specific sense primer P5 (Fig. 1*A*) and a common antisense primer (P10, 5′-GCTCGAGGAATCATAGTCTAGC-3′) located in the 3′-UTR (nucleotides 3647–3667) of the published HKα2 sequence (17). An *Xho*I site (underlined) was incorporated into the 5′ end of the primer to facilitate eventual subcloning into the mammalian expression vector pcDNA3.1−/Neo (Invitrogen). The amplicons were first subcloned into pCR2.1™ and sequenced on both strands. The sequence-verified encoding DNA for HKα2b was then excised from pCR2.1™ and cloned into the *Xba*I and *Xho*I sites of pcDNA3.1−/Neo downstream of the cytomegalovirus promoter. The resultant recombinant molecule was designated pcDNA3.1−/HKα2b-Neo.

Cloning of the Rat $HK\beta_g$ Subunit cDNA—The encoding DNA of the rat $HK\beta_g$ subunit was PCR-amplified from rat stomach cDNA using primers flanking the coding region: sense 5′- ATAAGCTTCAGCCCTGCAGGAGAAG-3′ (nucleotides +16 to +32 of the published sequence (25)) and antisense 5′- ATTCTAGATTACTTCTGTATTGTGAGC-3′ (nucleotides +878 to +896 of the published sequence). *Hin*dIII and *Xba*I sites (underlined) were added to the 5′ ends of the sense and antisense $HK\beta_g$ primers, respectively, to facilitate subcloning. The resultant amplicon was digested with *Hin*dIII and *Xba*I and subcloned into these sites of the mammalian expression vector pcDNA3.1+/Zeo (yielding the recombinant pcDNA3.1/$HK\beta_g$-Zeo). The insert $HK\beta_g$ DNA was sequenced to verify its authenticity.

Primer Extension—Antisense primers (Fig. 1*A*) specific for HKα2a (P7) and HKα2b (P8 and P9) were 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The primers were annealed to 10 μg of total RNA from distal colon at 58 °C for 20 min. After cooling at room temperature for 10 min, the primers were extended with avian myeloblastosis virus reverse transcriptase at 42 °C for 15 min in a reaction mixture containing 50 mM Tris-HCl, pH 8.3, at 42 °C, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dNTP, 0.5 mM spermidine, and 2.8 mM sodium pyrophosphate. The reactions were stopped by the addition of gel loading dye, and the samples were heated at 90 °C for 10 min. The primer extension products were resolved by electrophoresis on 8% acrylamide, 7 M urea polyacrylamide gels in TBE buffer. The sizes of the primer extension products were established by comparison with a sequence ladder generated by cycle sequencing with the ^{32}P-labeled primer used for each extension reaction and the HKα2 partial genomic DNA clone (see "Results") as template.

Analysis of Rat Genomic DNA—The 5′ end of the HKα2 gene was analyzed using the Rat PromoterFinder™ DNA Walking Kit (CLONTECH), which contains separate pools ("libraries") of uncloned, genomic DNA that have been predigested with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, or *Ssp*I and ligated to an oligonucleotide anchor (adapter primer 1). A nested PCR approach was employed. In the first round, aliquots of each "library" were amplified with adapter primer 1 and HKα2 primer P1, using a program of 94 °C × 25 s, 72 °C × 4 min for 7 cycles, 94 °C × 25 s, 67 °C × 4 min for 32 cycles, and 67 °C × 4 min for 1 cycle. After analysis of an aliquot of the PCR products on a 1.2% agarose gel, the remaining PCR products were diluted 1:50 in sterile deionized H_20 and subjected to a second round of PCR, using the nested adapter primer 2 and the nested HKα2 primer P2 (Fig. 1*A*) in a program of 94 °C × 25 s, 72 °C × 4 min for 7 cycles, 94 °C × 25 s, 67 °C × 4 min for 20 cycles, and 67 °C × 4 min for 1 cycle. The amplified products were separated by electrophoresis in a 0.9% agarose gel, subcloned into pCR2.1™, and sequenced on both strands by a cycle sequencing method.

RNA Isolation and Northern Analysis—Total RNA was extracted from selected tissues and renal parenchymal zones of normal and K^+-deprived rats using RNAzol B (Tel-Test). The samples were quantitated by spectrophotometry at 260 nm. Isoform-specific cDNAs of roughly comparable length (Fig. 1*A*) were generated by PCR from the cloned HKα2a and HKα2b cDNAs, using primer pairs P3 + P4 and P5 + P6 (Fig. 1*A*) directed at the unique 5′ exonic sequences of the HKα2a and HKα2b isoforms, respectively. Sequence analysis showed that these regions exhibited no significant homology to each other or to any sequence in the GenBank data base. A rat GAPDH cDNA (nucleotides 469–984, Ref. 26) was also generated by PCR. For Northern analysis,

the GAPDH and HKα2a- and HKα2b-specific cDNAs were radiolabeled with ^{32}P by the random primer method according to the manufacturer's instructions (Prime-a-Gene, Promega, Madison, WI). Fifteen μg of total RNA per lane were separated by size on 1% agarose, 2% formaldehyde gels and blotted to nylon membranes (Hybond N, Amersham Corp.). After UV cross-linking, the blots were visualized under UV light, hybridized for 2 h at 68 °C in QuickHyb solution (Stratagene) with probes specific for HKα2a, HKα2b, or GAPDH (as an additional control for RNA quality and equality of sample loading and transfer), and washed to a final stringency of 0.1 × SSC, 0.1% (s/v) SDS at 60 °C. Autoradiographs of the blots were prepared at −70 °C. In several experiments (as indicated in the figure legends), the blots were sequentially hybridized with HKα2a and HKα2b DNA probes of comparable size and specific activity, followed by the GAPDH DNA probe, with the blots being stripped before proceeding to the next analysis. After each stripping, autoradiographs of the blots were prepared to verify removal of the probe.

In Vitro Transcription and Translation—pcDNA3.1−/HKα2b-Neo and the HKα2a encoding DNA subcloned into the vector pAGA2 (16) were transcribed and translated in the presence of [^{35}S]methionine with T7 RNA polymerase and the TNT-coupled reticulocyte lysate kit (Promega, Madison, WI). The synthesized proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by fluorography.

Cell Culture and Transfection—HEK 293 cells were grown in modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 2 mM L-glutamine (complete medium). Subconfluent HEK 293 cells grown on 10-mm culture dishes were transfected with pcDNA3.1−/Neo (as a vector control) or pcDNA3.1/HKα2b-Neo with the Tfx-50 reagent (Boehringer Mannheim) to yield HEK-NEO and HEK-HKα2b cell lines, respectively. In brief, 10 μg of plasmid DNA and 22 μl of Tfx-50 reagent were mixed with 6 ml of modified Eagle's medium. The mixture was added to the monolayers and incubated for 2 h at 37 °C in a 5% CO_2 incubator. Twelve ml of prewarmed complete medium was then overlaid onto the medium, and the cells were returned to the incubator. After 48 h, the medium was replaced with complete medium containing 600 μg/ml G418 (Life Technologies, Inc.). The G418-containing medium was replaced every 3 days until individual resistant colonies were isolated and established in culture as individual lines. All lines were maintained in G418 medium and frozen after one to three *in vitro* passages. HEK-HKα2b clone 25 was used in the functional analysis detailed below. To test whether coexpression of the $HK\beta_g$ affected functional expression of HKα2b, HEK-NEO and HEK-HKα2b cell lines were stably transfected with pcDNA3.1/$HK\beta_g$-Zeo and selected in complete medium containing 600 μg/ml G418 and 250 μg/ml Zeocin. Cells surviving selection were screened for HKα2b and/or $HK\beta_g$ expression by Northern analysis with probes specific for each subunit. The doubly transfected cells were termed HEK-HKα2b/$HK\beta_g$, and clone 40 was selected for further functional analysis.

$^{86}Rb^+$ Uptake—Uptake of $^{86}Rb^+$, a K^+ congener, was measured at 37 °C in transfected HEK 293 cells grown in 24-well plates according to a published protocol (22). Monolayers were rinsed five times and preincubated in uptake buffer (145 mM NaCl, 1 mM KCl, 10 mM glucose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM NaH_2PO_4, 32 mM HEPES, pH 7.4, and 200 μM bumetanide) at 37 °C for 20 min in the presence or absence of different concentrations of ouabain as indicated in the figure legends. External 1 mM K^+ was used in these assays, because K^+ competitively inhibits both Sch 28080 and ouabain binding to X^+-K^+-ATPase α subunits, and this concentration is within the narrow range of K_m values reported for K^+ dependence of all known X^+-K^+-ATPase α subunits. Uptake was initiated by adding 0.2 ml of uptake buffer containing ~4 μCi/ml $^{86}Rb^+$. After 12 min, the reaction was stopped by six rapid washes with ice-cold stop buffer (100 mM $MgCl_2$, 10 mM Tris-HEPES, pH 7.4). Parametric studies indicated that this time point was in the linear range of uptake. The cells were solubilized in 2% SDS, 0.1 N NaOH, and the resulting extracts were measured for $^{86}Rb^+$ by Cerenkov radiation and for protein content by the BCA Protein Assay Reagent (Pierce). Triplicate or quadruplicate measurements were obtained in each uptake condition.

Data Analysis—The intensities of bands on the Northern blot autoradiograms were measured by whole band densitometry software running on a SPARC Station IPC (Sun Microsystems, Mountain View, CA) equipped with an image analysis system (BioImage, Ann Arbor, MI). Predictions of membrane-spanning regions and their orientation were generated by the TMpred program (27) through the ISREC Bioinformatics Group server. Predictions of potential promoter regions were obtained with a neural networks algorithm (28) through the LBNL Human Genome Informatics Group server. Potential regulatory motifs in the HKα2 gene were identified with Transcription Element Search Software from the Computational Biology and Informatics Laboratory server of the University of Pennsylvania School of Medicine, using the Transfac 3.1 data base. Quantitative data are presented as mean ± S.E. and were analyzed for significance by analysis of variance. Significance was assigned at $p < 0.05$.

RESULTS

cDNA Cloning and Structural Analysis of a Truncated N-terminal Variant of the H^+-K^+-ATPase α2 Subunit—The anchor-ligated cDNAs synthesized from rat kidney mRNA were subjected to 5′-RACE using adapter primer 2 and HKα2-specific primer P1 from exon 2 (Fig. 1*A*). Two distinct PCR products of ~400 and ~600 bp, subsequently shown to correspond to the 5′ ends of HKα2a and HKα2b, respectively, were consistently amplified. These products were isolated, subcloned, and sequenced. A total of 16 RACE reactions for both amplicons was analyzed in this manner. The two RACE product subtypes differed in sequence at their 5′ ends but were identical at their 3′ ends, with common sequence beginning at the codon for Lys^4 of the known HKα2a sequence (Fig. 1*A*). HKα2a was identical in sequence to the corresponding region of the HKα2 cDNA reported by Crowson and Shull (17) but included an additional 72 bp at its 5′ end, so that the total 5′-UTR was 274 bp.

By using a sense primer from the 5′-UTR of HKα2b and an antisense primer derived from the 3′-UTR of the published HKα2 cDNA (17), a 3847-bp cDNA, including the entire HKα2b coding region, was PCR-amplified from rat kidney cDNA, subcloned, and sequenced. The HKα2b sequence was identical to that of HKα2a beginning at the codon for Lys^4 of HKα2a (Fig. 1*A*). The first AUG triplet of the HKα2b mRNA that resides within a favorable context for translation initiation corresponds to Met^{109} of HKα2a. Thus the predicted HKα2b peptide of 929 amino acids (mass = 102,554 Da) lacks the first 108 amino acids of the HKα2a sequence (1036 amino acids, mass = 114,966 Da), which includes consensus sites for cAMP phosphorylation (Thr^5) and protein kinase C phosphorylation (Ser^{78}) (Figs. 1*A* and 2). Secondary structure models of the HKα2a and HKα2b deduced amino acid sequences predict that HKα2b would have a shorter N-terminal cytosolic segment but would otherwise share identical topology to HKα2a (Fig. 2).

As predicted from the sequence analysis, *in vitro* transcription and translation of HKα2b cDNA yielded an ~104-kDa protein, whereas *in vitro* transcription and translation of HKα2a cDNA yielded ~118- and ~104-kDa proteins (Fig. 3). The latter result indicates that both HKα2 variant proteins can be translated *in vitro* from HKα2a mRNA by utilization of the first and second in-frame AUG codons.

Genomic Organization of the 5′ End of the HKα2 Gene and Mapping of the Transcription Start Sites—Analysis of the HKα2a and HKα2b 5′-RACE products suggested that both mRNAs are derived from a single gene by utilization of alternative splice sites at the 5′ region. To determine the order of the exons and the intervening genomic sequences, we used nested reverse primers derived from the 5′ region common to both variants and adapter-ligated rat genomic DNA libraries to PCR amplify a portion of the 5′ region of the HKα2 gene. PCR products of ~1.6- and 0.5-kb were consistently amplified from the *Dra*I and *Pvu*II libraries, respectively. These amplicons were subcloned into pCR2.1™ and sequenced on both strands. Sequence analysis indicated that the HKα2b transcript is the product of an alternative transcription initiation site located within the first intron (Fig. 1, *A* and *B*). The 5′ end of the HKα2b mRNA represents a 5′ extension of exon 2 that is excised in the HKα2a mRNA. In support of this construct, a consensus 5′ splice donor site (5′-GTGAGT-3′) was identified at the exon 1/intron 1 boundary, and a consensus 3′ acceptor site

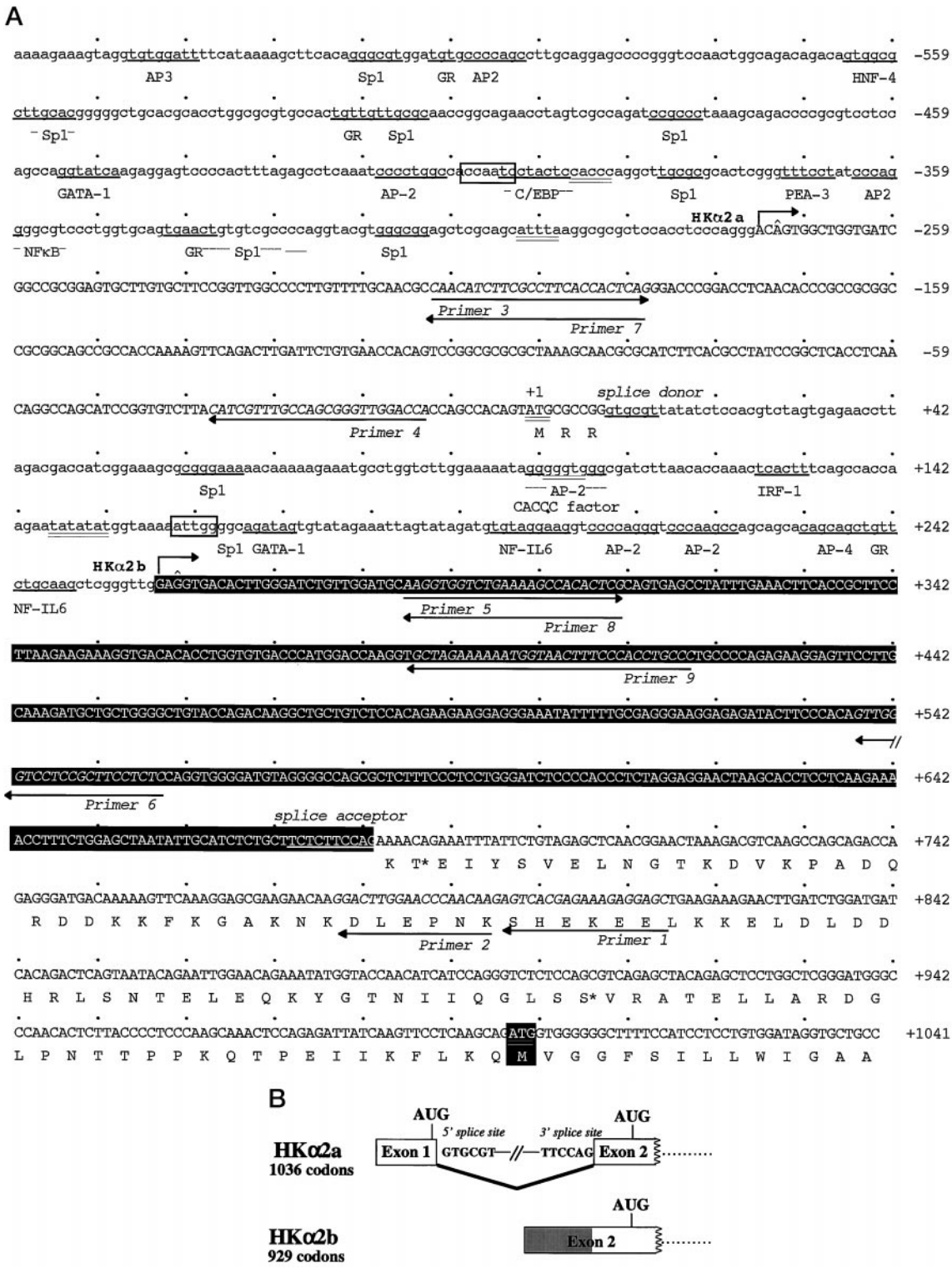

FIG. 1. **5′ end of the rat H^+-K^+-ATPase α2 (HKα2) gene.** *A*, nucleotide sequence, potential elements, and PCR primer sequences. (In the discussion and Fig. 1, we have assumed that the 5′-most exon in the H^+-K^+-ATPase α2 gene is exon 1. However, the possible existence of additional upstream exons has not been rigorously excluded.) The 5′-flanking regions and intron 1 sequences are indicated by *lowercase letters*. The sequences of the HKα2a and HKα2b transcription units are indicated in *uppercase black* and *white letters*, respectively. The 5′ splice donor and 3′ splice acceptor sites are identified. Amino acids encoded by exons 1 and 2 are indicated *below* the appropriate codons. Nucleotides used to generate oligonucleotide primers for PCR and primer extension are *italicized*, and their orientation (sense, antisense) is indicated by *arrows*. Potential TATA and CACCC sequences are *doubly underlined*, and CCAAT sequences are indicated by *open boxes*. The transcription initiation sites defined by primer extension are designated by *arrows*. 5′-Most ends of the 5′-RACE sequences are indicated by a *caret* over the nucleotide. Sequences exhibiting homology to *cis*-elements or transcription factor binding sites (AP-2, AP-3, Sp1, GATA-1, NF-κB, PEA-3, C/EBP, GR, IRF-1, NF-interleukin 6, and HNF-4) are *underlined*. *Numbers* to the *right* of the figure indicate nucleotide positions relative to the HKα2a translation start site. Potential sites of protein kinase A and protein kinase C phosphorylation are designated by *asterisks*. *B*, schematic representation showing alternative splicing of HKα2a and HKα2b mRNAs. Exons are depicted as *boxes*, with the *shaded area* representing the sequence spliced from the HKα2a mRNA and retained in the HKα2b sequence. The translation initiation codons of the major open reading frames are indicated by *AUG*. The positions of the exon groups and splicing patterns were identified by a PCR cloning method as detailed under "Experimental Procedures." The putative first promoter precedes exon 1, which contains a start ATG and only 3 codons. The putative second promoter resides in intron 1.

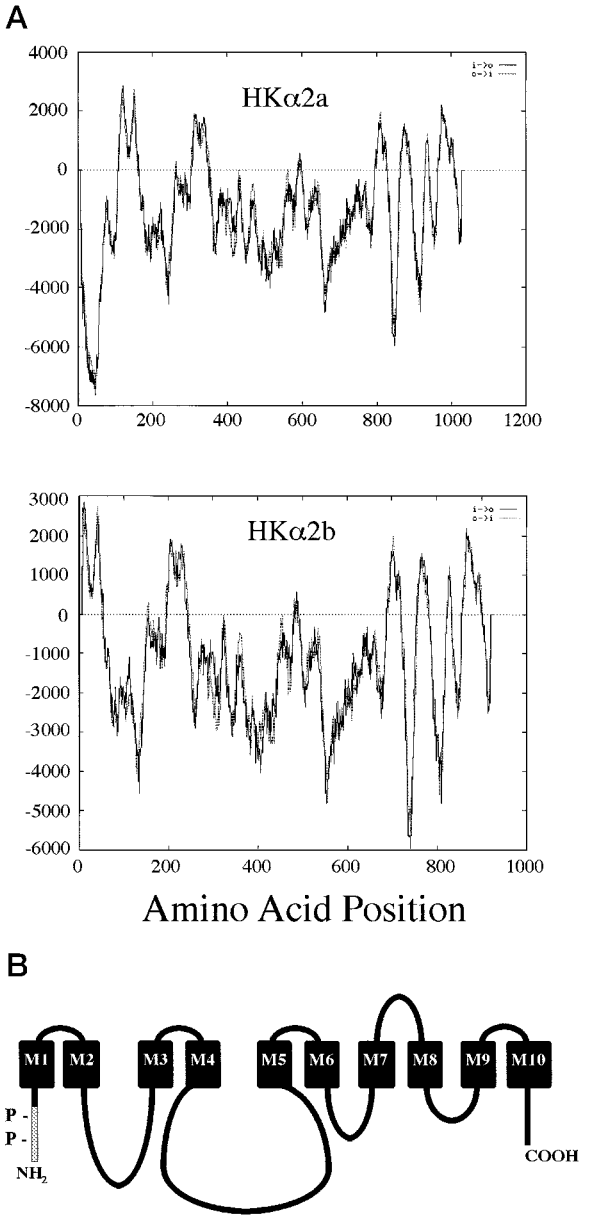

FIG. 2. **Secondary structure analysis and predicted structural models of H⁺-K⁺-ATPase α2a (HKα2a) and H⁺-K⁺-ATPase α2b (HKα2b) isoforms.** *A*, TMpred analysis (27) of the HKα2a and HKα2b amino acid sequences using a window of 17–33 amino acids for the length of the hydrophobic part of the transmembrane helix. *B*, schematic representation of the membrane topology of HKα2a and HKα2b. The truncated portion for HKα2b is indicated by *shading*. Putative protein kinase A and protein kinase C phosphorylation sites are indicated by *P*.

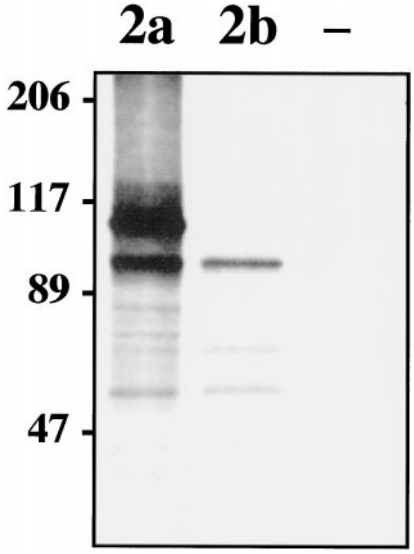

FIG. 3. ***In vitro* transcription and translation of H⁺-K⁺-ATPase α2a (HKα2a) and H⁺-K⁺-ATPase α2b (HKα2b).** Fluorograph of SDS-10% polyacrylamide gel of peptides generated, as described under "Experimental Procedures," by *in vitro* transcription and translation of HKα2a and HKα2b encoding DNAs in the presence of [^{35}S]methionine. −, indicates no DNA template. Molecular weights were determined by comparisons of their mobilities to known standards. Results are representative of two experiments.

(CAG) (29) was found in the expected 5′ region of exon 2 (Fig. 1*A*).

The transcription initiation sites for the two mRNAs were mapped by primer extension analysis of total RNA from distal colon. A single major extension product was observed for both the HKα2a and HKα2b reactions (Fig. 4), and these corresponded within 2 to 3 nucleotides to the 5′-most ends of the 5′-RACE products from rat kidney cDNA. The size of the HKα2a primer extension product places the transcription initiation site 274 bp upstream of the initiation methionine codon, the first ATG triplet 3′ to the transcription start site (Fig. 1*A*). The nucleotide sequences surrounding the HKα2a transcription initiation site closely matches a CAP site consensus sequence (30). The putative transcription initiation site for HKα2b resides 424 bp upstream of the exon 1/exon 2 alternative splice junction. The total length of the HKα2b 5′-UTR is 739 bp, and the length of the mRNA characterized is 3874 bp. Interestingly this 5′-UTR region contains eight upstream open reading frames (uORFs) as follows: 1) +24 to 68; 2) +117 to +446; 3) +139 to +315; 4) +189 to +446; 5) +489 to +560; 6) +579 to +773; 7) +618 to +773; and 8) +678 to +773 (numbering with +1 at putative HKα2b transcription start site).

Analysis of Potential Gene Control Elements—The HKα2 partial genomic clone contained sequences of ~380 and ~205 bp immediately 5′ to the transcription start sites of the HKα2a and HKα2b transcription units, respectively. These sequences share no obvious homology, and they were examined for potential DNA elements that may contribute to transcriptional initiation and regulation. The HKα2a 5′-flanking region contains a TATA-like sequence (ATTTAA), a CACCC sequence (31), and a CCAAT motif (30) beginning 27, 127, and 133 bp, respectively, 5′ to the transcription start site, which likely comprise the core promoter module (Fig. 1*A*). The 380 bp immediately preceding the transcription start sites contains several potential *cis*-elements that may serve as binding sites for transcription factors. These include 7 Sp 1 sites (32), 3 AP-2 sites (33), 2 GR sites (31), and single GATA-1 (34), C/EBP (35), PEA-3 (36), NF-κB (37), and HNF-4 (38) motifs (Fig. 1*A*).

The region 5′ to the transcription start site of HKα2b contains potential promoter elements, including a TATATAT motif, a reverse complement of a CCAAT sequence, and a CACCC sequence 74, 61, and 120 bp, respectively, upstream of the putative transcription start site. Two Sp1 sites, two AP-2 sites, and single sites for NF-interleukin 6 (39), IRF-1 (40), and GATA-1 were identified in the 5′-flanking region of the HKα2b transcription unit (Fig. 1*A*).

HKα2 Isoform mRNAs Are Expressed in Colon and Kidney—Northern blots of total RNA harvested from an array of tissues harvested from K⁺-replete rats were probed with ^{32}P-labeled DNA probes specific for each HKα2 subtype (Figs. 5 and 6). Both isoforms were expressed prominently in the distal colon (Fig. 5) and very weakly in the proximal colon and normal kidney (Fig. 6). No transcripts were detected in skeletal mus-

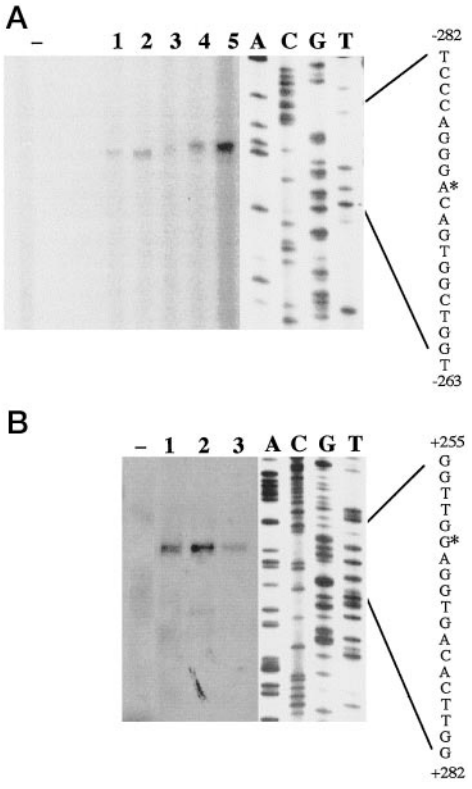

FIG. 4. **Primer extension analysis of 5′ ends of H⁺-K⁺-ATPase α2 isoform mRNAs.** Primer extension experiments were performed with ^{32}P-labeled HKα2 isoform-specific oligonucleotide primers P7 (HKα2a) and P8 and P9 (HKα2b, see Fig. 1*A*), respectively, and total RNA from rat distal colon as described under "Experimental Procedures." Representative autoradiographs for the HKα2a and HKα2b (primer P9 results) results are shown in *A* and *B*, respectively. Identical mapping results were obtained with the HKα2b primers P8 and P9. Yeast tRNA served as a negative control (−). *Lanes 1–5* represent RNA samples obtained from 5 different rats. *Lanes A, C, G,* and *T* are sequencing reactions on the same gel using the same primer and the plasmid construct bearing the HKα2 1.6-kb genomic fragment obtained from the *Dra*I-digested rat genomic DNA library (see "Experimental Procedures"). The base corresponding to the major transcription start site for each isoform is labeled by an *asterisk* within the genomic DNA sequence shown to the *right*. The slightly slower mobility of the primer extension products in *lanes 3–5* of *A* reflects a slight delay in loading of these samples. In both figures, short and long exposures of the film were used to allow optimal comparison of the sequencing ladders with the primer extension reactions (which were run on the same gel). All experiments were performed in triplicate.

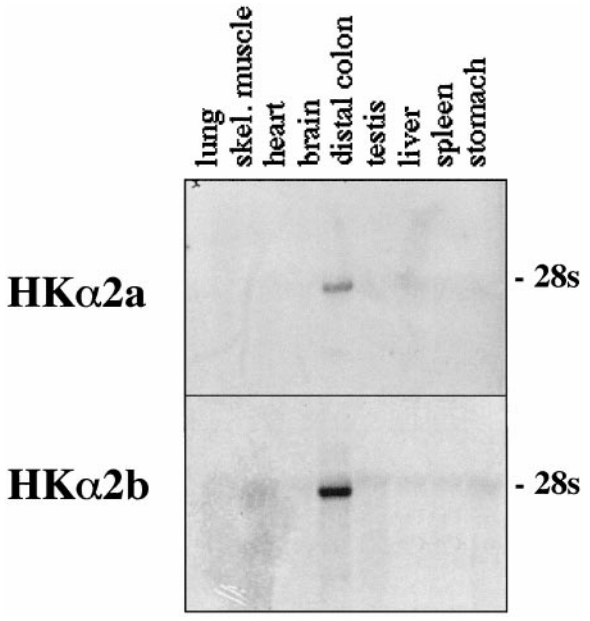

FIG. 5. **Tissue distribution of H⁺-K⁺-ATPase α2a (HKα2a) and H⁺-K⁺-ATPase α2b (HKα2b) mRNA in rat by high stringency Northern analysis.** Total RNA (15 μg) from the indicated rat tissues was electrophoresed in 1% agarose-formaldehyde gels and transferred to nylon membranes as detailed under "Experimental Procedures." The filters were probed sequentially with ^{32}P-labeled DNA probes specific for HKα2a and HKα2b (see Fig. 1 and "Experimental Procedures").

cle, heart, brain, stomach, spleen, liver, testis, or lung (Fig. 5), even with prolonged autoradiographic exposures. Failure to detect transcripts in these latter tissues also indicates that the HKα2 isoform-specific probes did not cross-hybridize with the four known Na⁺-K⁺-ATPase α subunit isoforms (abundantly expressed in heart, brain, skeletal muscle, and/or testis (41, 42)) or the HKα1 subunit (abundantly expressed in stomach (14)). Moreover, reprobing the blots with a ^{32}P-labeled DNA probe for GAPDH indicated comparable abundance and integrity of the blotted RNA samples (data not shown).

Expression of HKα2 Subunit Isoform mRNAs in Kidney and Colon of Control and K⁺-restricted Rats—To determine the response of the HKα2a and HKα2b gene products to dietary K⁺ restriction in rat kidney and colon, Northern analysis with ^{32}P-labeled DNA probes specific for each HKα2 subtype was performed on total RNA harvested from the proximal colon, distal colon, and renal cortex, outer medulla, and inner medulla of control and K⁺-restricted rats. In control rats, an abundant ~4.0-kb subunit transcript (HKα2b ≫ HKα2a) was detected with both the HKα2a- and HKα2b-specific probes in distal colon (Fig. 6*A*). In addition, the HKα2b-specific probe hybridized to a much less abundant ~6.0-kb transcript in distal colon (Fig. 6*A*). It is not known whether this larger transcript represents a processing intermediate or an mRNA with an alternate polyadenylation signal, but similar results were reported by Crowson and Shull (17), who used C-terminal coding and 3′-UTR sequences as probes. With prolonged autoradiographic exposures (3 days), very low, comparable levels of HKα2a subunit mRNA were detected in the proximal colon, cortex, and outer and inner medulla (data not shown). When these blots were reprobed with the HKα2b-specific probe of roughly comparable size and specific activity, detectable ~4.0-kb transcripts were observed in the same structures after overnight exposure, suggesting that HKα2b is expressed at higher levels, albeit still very low, than HKα2a in normal kidney and colon.

To determine whether the relative levels of the HKα2a or HKα2b subunit mRNAs in kidney and colon varied with body K⁺ balance, Northern blots of total RNA isolated from control and K⁺-restricted rats (n = 4 for each group) were probed sequentially with the subtype-specific probes of comparable size and specific activity. Autoradiographs of the blots prepared after 3 days of film exposure (to allow detection of HKα2a mRNA in controls) were analyzed by scanning densitometry. The K⁺-restricted rats exhibited greater levels of both HKα2a and HKα2b in the cortex and outer and inner medulla compared with controls (Fig. 6*A*). The two subtypes appeared to be coordinately up-regulated in the kidney zones of K⁺-restricted rats, but accurate quantitation of the degree to which expression was enhanced with chronic K⁺ deprivation was not possible because of the low basal expression of both mRNAs in the kidney. For each K⁺-restricted animal, the abundance of HKα2b mRNA was greater than that of HKα2a in each renal parenchymal zone (Fig. 6*B*), although the magnitude of the difference was highly variable. In contrast to kidney, neither the HKα2a nor HKα2b transcript abundance in proximal or distal colon differed between control and K⁺-restricted rats

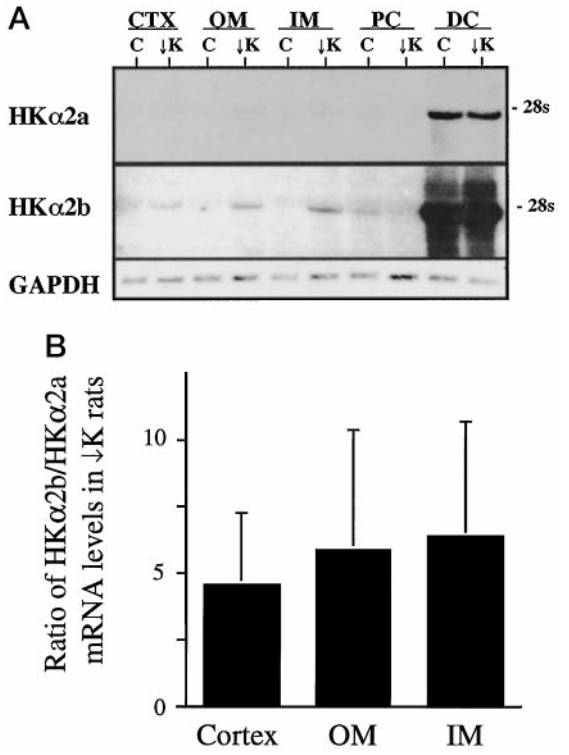

FIG. 6. **H^+-K^+-ATPase α2a (HKα2a) and H^+-K^+-ATPase α2b (HKα2b) subunit mRNA expression in colon and renal parenchymal zones of control (C) and potassium-restricted (↓) rats.** *A*, representative Northern blot of total RNA from cortex (*CTX*), outer medulla (*OM*), inner medulla (*IM*), proximal colon (*PC*), and distal colon (*DC*) isolated from control and K^+-restricted rats ($n = 4$ animals for each group). The filters were probed sequentially with ^{32}P-labeled DNA probes specific for HKα2a, HKα2b, and GAPDH as described under "Experimental Procedures." Autoradiographic exposure was overnight. *B*, histogram showing results of densitometric analysis of Northern blots. The ratio of the relative optical density of the HKα2b and HKα2a transcript bands in the K^+-restricted rats is plotted.

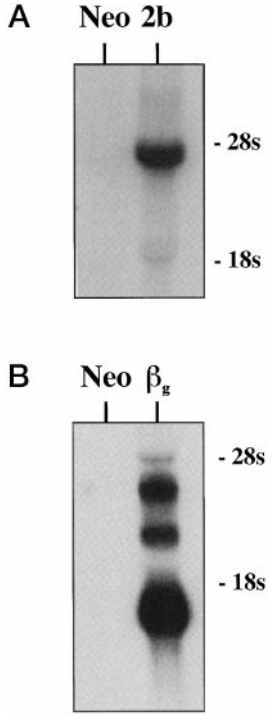

FIG. 7. **Heterologous expression of the H^+-K^+-ATPase α2b subunit with or without the gastric H^+-K^+-ATPase β subunit (HKβg) in HEK 293 cells.** *A*, autoradiograph of representative Northern blots of total RNA harvested from HEK-NEO cells (*NEO*) and HEK-HKα2b (*2b*) clone 25 cells. The blot was probed with a ^{32}P-labeled DNA probe specific for HKα2b. *B*, autoradiograph of Northern blot of total RNA harvested from HEK-NEO and HEK-HKα2b/HKβg (*βg*) clone 40 cells. The latter cell line was generated by stable transfection of HEK-HKα2b clone 25 cells with the DNA encoding HKβg. The blot was probed with a ^{32}P-labeled DNA probe specific for HKβg. The minor, higher molecular weight bands presumably represent processing intermediates or differences in polyadenylation (provided by the bovine growth hormone poly(A) sequence included in the pcDNA3.1+/Zeo vector) of the HKβg mRNA.

(Fig. 6*A*).

Functional Expression of the H^+-K^+-ATPase α2b Subunit in HEK 293 Cells—A dual selection strategy, using separate mammalian expression vectors containing the encoding DNAs for HKα2b and HKβg together with the neomycin and Zeocin resistance genes, respectively, was employed to generate cell lines stably expressing the HKα2b subunit, the HKβg subunit, or both subunits. HEK 293 cells were chosen as the recipient cells for the transfection experiments because they are easily transfected, do not express H^+-K^+-ATPase α or β subunit gene products, their endogenous Na^+-K^+-ATPase is highly sensitive to ouabain (22), and they permit analysis of H^+-K^+-ATPase biosynthesis and subunit assembly in mammalian cells at 37 °C (a factor that has been suggested to influence the fidelity of oligomerization and membrane insertion of the pump, Ref. 22).

Northern analysis revealed that cells stably transfected with the DNA encoding HKα2b (HEK-HKα2b cells) expressed the expected ~4.0-kb mRNA recognized by the HKα2b probe (Fig. 7*A*). The HEK-NEO and HEK-HKα2b cells were then stably transfected with the HKβg cDNA. Northern analysis revealed that the resulting HEK-HKβg cells (data not shown) and the HEK-HKα2b/HKβg cells expressed the ~1.4-kb transcript expected for the HKβg mRNA containing the bovine growth hormone poly(A) tail provided by the pcDNA3.1+/Zeo vector (Fig. 7*B*). In contrast, HEK-NEO cells exhibited neither HKα2b (Fig. 7*A*) nor HKβg (Fig. 7*B*) gene expression.

As an initial characterization of the functional properties of the HKα2b subunit, untransfected HEK 293, HEK-NEO, HEK-HKα2b, and HEK-HKα2b/HKβg cells were grown in media containing 1 μM ouabain. Only the HEK-HKα2b/HKβg cell lines survived ouabain treatment, suggesting that the fully assembled HKα2b/HKβg pump can compensate for an inoperative Na^+-K^+-ATPase in maintaining the intracellular ionic milieu, as has been reported for the ATP1AL1/HKβg pump (22). $^{86}Rb^+$ uptake of HEK-NEO, HEK-HKβg, HEK-HKα2b clone 25, and HEK-HKα2b/HKβg clone 40 cell lines was assayed to determine whether the truncated variant could be expressed in the plasma membrane to conduct active K^+ uptake. Bumetanide was included in the incubation medium to inhibit K^+ entry via the Na^+-K^+-$2Cl^-$ transporter. The basal rate of uptake, measured in the absence of ouabain, was comparable among the different cell lines, with the exception of the HEK-HKα2b clone 25 cells, whose basal uptake was ~20% less ($p < 0.05$) than the other transfectants: (in nmol/mg protein/min; $n = 3$ for each) HEK-NEO, 4.8 ± 0.05; HEK-HKβg, 4.2 ± 0.06; HEK-HKα2b, 3.3 ± .01; HEK-HKα2b/HKβg, 4.2 ± 0.7. As seen in Fig. 8*A*, the endogenous Na^+-K^+-ATPase of the wild-type HEK 293 and HEK-NEO cells was quite sensitive to ouabain inhibition as follows: 1 μM inhibited ~97% of the total $^{86}Rb^+$ uptake, and 1 mM ouabain virtually abolished uptake in the presence of external 1 mM K^+. Similar sensitivity to ouabain inhibition was observed in HEK-HKβg cells (Fig. 8*A*). In contrast, the HEK-HKα2b clone 25 and HEK-HKα2b/HKβg clone 40 cell lines were less sensitive to 1 μM ouabain, exhibiting uptakes that were ~3.5- and 5-fold greater, respectively, than the HEK-NEO control (Fig. 8*A*). In the presence of 1 mM oua-

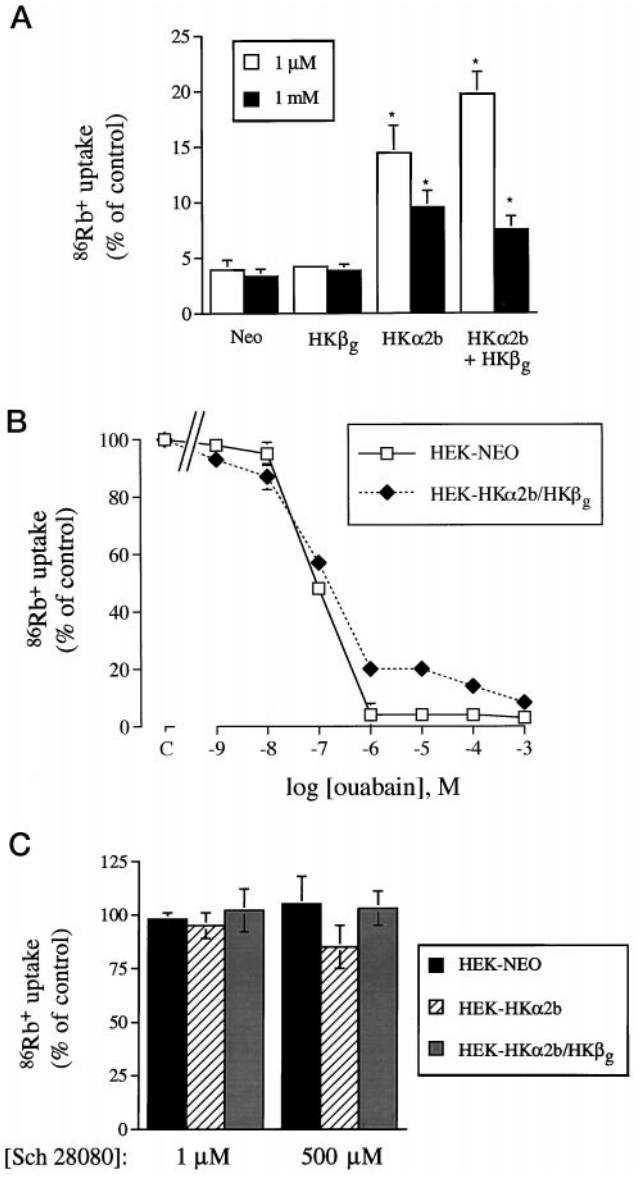

FIG. 8. **Effect of ouabain and Sch 28080 concentration on $^{86}Rb^+$ uptake in HEK 293 cells stably expressing the H^+-K^+-ATPase α2b subunit with or without the gastric H^+-K^+-ATPase β subunit ($HK\beta_g$) subunit.** *A*, $^{86}Rb^+$ uptake in HEK-NEO, HEK-$HK\beta_g$, HEK-HKα2b clone 25, or HEK-HKα2b/$HK\beta_g$ clone 40 cells was determined in the presence of 1 mM KCl, 200 μM bumetanide, and 1 μM or 1 mM ouabain. Data (means ± S.E.) are expressed as the percent of the control value (presented under "Results") measured in the absence of ouabain ($n = 3$). *, $p < 0.05$ compared with comparably treated HEK-NEO and HEK-$HK\beta_g$ cells. The apparent difference between the HEK-HKα2b clone 25 and the HEK-HKα2b/$HK\beta_g$ clone 40 cells did not achieve statistical significance. *B*, $^{86}Rb^+$ uptake in HEK-NEO and HEK-HKα2b/$HK\beta_g$ clone 40 cells was measured as a function of the ouabain concentration in the presence of 1 mM KCl and 200 μM bumetanide. Data (means ± S.E.) are expressed as the percent of the control value measured in the absence of ouabain ($n = 3$). Where error bars are not seen, they are contained within the datum point. Control values were 4.9 ± 0.3 nmol/mg protein/min for HEK-NEO cells and 4.5 ± 0.5 nmol/mg protein/min for HEK-HKα2b/$HK\beta_g$ clone 40 cells. *, $p < 0.05$ compared with HEK-NEO cells. *C*, $^{86}Rb^+$ uptake in HEK-HKα2b cells and HEK-HKα2b/$HK\beta_g$ clone 25 was determined in the presence of 1 mM KCl, 200 μM bumetanide, 1 μM ouabain (to inhibit endogenous Na^+-K^+-ATPase activity), and vehicle (control) or the indicated concentrations of Sch 28080 (means ± S.E. $n = 3$).

bain, $^{86}Rb^+$ uptake by the HEK-HKα2b clone 25 and HEK-HKα2b/$HK\beta_g$ clone 40 cell lines was roughly 1.5- to 2-fold greater than the HEK-NEO controls (Fig. 8*A*). Dose-response curves for ouabain inhibition of $^{86}Rb^+$ uptake (Fig. 8*B*) confirmed that the HKα2b/$HK\beta_g$ clone 40 cells contributed two components of $^{86}Rb^+$ uptake: one that was extremely sensitive to ouabain (the endogenous Na^+-K^+-ATPase) and one that was intermediate in its sensitivity to ouabain (the HKα2b pump). Assuming, then, that the $^{86}Rb^+$ uptake mechanism that operates in the presence of ≥1 μM ouabain in these cells represents the contribution of the HKα2B pump, the approximate IC_{50} (IC_{50}, concentration of inhibitor causing 50% inhibition of corresponding $^{86}Rb^+$ uptake) for ouabain inhibition of $^{86}Rb^+$ uptake for the HKα2B pump was ~400 to 800 μM in the presence of external 1 mM K^+ (Fig. 8*B*).

The effects of Sch 28080, a potent inhibitor of the HKα1 subunit (16), were tested on the component of $^{86}Rb^+$ uptake insensitive to 1 μM ouabain. Sch 28080, at concentrations up to 500 μM, had no statistically significant effect on $^{86}Rb^+$ uptake in the HEK-NEO, HEK-HKα2b clone 25, and HEK-HKα2b/$HK\beta_g$ clone 40 cell lines (Fig. 8*C*). This insensitivity to Sch 28080 was also observed in studies of the full-length HKα2 subunit expressed in heterologous systems (16, 18, 19).

DISCUSSION

Analysis of the regulation of active K^+ reabsorption in the renal collecting duct and distal colon has been hampered by the lack of structural data concerning potential control mechanisms governing HKα2 gene expression. In this study, we characterized two alternatively spliced products of the rat HKα2 gene, HKα2a and HKα2b, that apparently arise from the use of alternative promoters and differ in the length of their N termini and their relative abundance in kidney and colon. Heterologous expression studies of the novel transcript in HEK 293 cells indicate that HKα2b encodes a plasma membrane mechanism for K^+ uptake that, like that of the full-length HKα2 subunit (16, 18, 19), is relatively Sch 28080-resistant, intermediate in its sensitivity to ouabain, and operates more effectively when coexpressed with the $HK\beta_g$ subunit. The HKα2b isoform represents the most abundantly expressed HKα2 transcript in the rat kidney and distal colon and the principal H^+-K^+-ATPase transcript up-regulated in the renal medulla of K^+-deprived rats. We also identified structural features that may govern transcriptional initiation and control as well as translational regulation of these isoforms. Our results suggest that both HKα2 isoforms may contribute to K^+ conservation during chronic hypokalemia, and they uncover a new degree of regulatory complexity for the X^+-K^+-ATPase α subunit gene family.

The first variant, HKα2a, is the previously described (17) 1036-amino acid protein, which is encoded by a 4.0-kb mRNA transcribed from the 5′-most putative promoter. Primer extension analysis places the major transcription initiation site 274 bp upstream of the translation initiation methionine. Exon 1 includes the 5′-UTR and encodes the first three amino acids of the primary HKα2a translation product. This structural theme is common to other members of the X^+-K^+-ATPase α subunit family. The analogous exon in *ATP1AL1* also encodes 3 amino acids, whereas those for the HKα1, and Na^+-K^+-ATPase α1 and α2 subunits genes encode 4 amino acids, and that for the Na^+-K^+-ATPase α3 subunit gene encodes only 2 amino acids. The 5′-flanking region of HKα2a contains common basal promoter elements. An AT-rich sequence that might serve as a TATA element begins 21 bp 5′ to the transcription start site. This sequence is preceded by potential CCAAT (30) and CACCC (31) elements residing within the preferred context for such elements. In addition, sequence inspection of the 5′-flanking regions revealed potential *cis*-acting DNA elements, including sites for AP-2, AP-3, GATA-1, HNF-4, C/EBP, GR, PEA-3, NF-κB, and multiple Sp1 sites sequences that may participate in transcriptional regulation of this gene. Of these, Sp1 (43) and GATA DNA-binding proteins (44) have been shown to play

important roles in transcriptional activation of the HKα1 gene. Since we did not confirm the 5′ end of the HKα2 gene, other potential regulatory elements may reside upstream of the sequence we characterized.

The second variant, HKα2b, has not been previously recognized. This 929-amino acid protein is also encoded by an ~4.0-kb mRNA that is transcribed from an internal putative promoter residing in intron 1. Given the near-identical size of the major HKα2a and HKα2b mRNA transcripts, Northern analysis with probes directed to sites distal to the alternative splice site in exon 2 would be unable to distinguish between the two isoforms. Our DNA sequence data (Fig. 1*A*) combined with the *in vitro* transcription and translation results (Fig. 3) and functional expression data (Fig. 7, *B* and *C*) indicate that the HKα2b isoform encodes a protein with the requisite features of an X^+-K^+-ATPase α subunit. Primer extension and 5′-RACE identified a putative site for HKα2b transcription initiation, but given the context of the surrounding nucleotides, additional transcription start sites may be located upstream of the site we identified (that is closer to the TATA, CACCC, and reverse complement CCAAT sequences found in the 5′-flanking region of the HKα2b transcription unit). Other potential *cis*-elements, including multiple AP-2 and Sp-1 sites, as well as consensus NF-interleukin 6, IRF-1, AP-4, GR, and GATA-1 sequences, were identified in this region. Conclusive evidence for the functional activity of the HKα2a and HKα2b promoter elements will require formal testing with promoter-reporter gene constructs.

A notable feature of the HKα2b mRNA is the complex 5′-UTR containing multiple, partially overlapping AUG triplets in ORFs upstream (uORF) of the translation initiation site of the major ORF. Recent analyses have shown that such uORFs are present in <10% of vertebrate mRNAs (45) and that in some instances they inhibit translational initiation at the major ORF. For example uORFs in the 5′-UTR of the retinoic acid receptor β2 and transforming growth factor β3 mRNAs dramatically inhibited CAP-dependent translation *in vitro* (46, 47). Moreover, studies of the retinoic acid receptor β2 mRNA in transgenic mice indicate a role for uORFs in tissue-specific and developmentally regulated gene expression (48). The relatively low level of $^{86}Rb^+$ uptake activity attributable to the HKα2b pump in the HEK-HKα2b and HEK-HKα2b/HKβ_g cell lines (Fig. 8*A*) despite abundant mRNA expression (Fig. 7*A*) might reflect this regulatory constraint. Alternatively, preference of HKα2b for an X^+-K^+-ATPase β subunit other than the endogenous Na^+-K^+-ATPase β1 subunit expressed in HEK 293 cells might limit expression of transport activity. The fact that coexpression of the HKβ_g subunit supported higher rates of $^{86}Rb^+$ uptake activity and was required for survival in 1 μM ouabain supports this latter hypothesis. Since studies of the full-length HKα2 subunit, expressed by cRNA injection in *Xenopus* oocytes, indicated that the rat Na^+-K^+-ATPase β1 and HKβ_g subunit support comparable rates of K^+ uptake (16), it remains to be determined whether the two HKα2 isoforms differ in their promiscuity for X^+-K^+-ATPase β subunits.

The functional and regulatory significance of the N-terminal truncation of HKα2b remains to be explored in further detail. The N terminus is the most variable structural region among the X^+-K^+-ATPase α subunits. Conceivably the decision to code for the N-terminal 108 amino acids present in HKα2a could dictate isoform-specific differences in membrane targeting or cytoskeletal association in polarized epithelia, regulation by protein kinase C or protein kinase A phosphorylation (since these sites are present in HKα2a but not HKα2b), ion transport kinetics, or inhibitor sensitivities. There is precedent for alternative promoters to direct the coding of protein variants that are targeted to different intracellular locales. The two variants of leukemia inhibitory factor, which exist as diffusible and extracellular matrix-associated isoforms, represent such an occurrence (49). The possibility for functional and pharmacological differences in the HKα2 isoforms is particularly intriguing since N-terminal deletion mutants of the closely related Na^+-K^+-ATPase exhibited altered K^+ deocclusion kinetics compared with wild-type pumps (50), and since the N-terminal truncation of HKα2b impinges on the H1-H2 domains, which have been implicated in ouabain and Sch 28080 binding to other X^+-K^+-ATPase α subunits (reviewed in Ref. 13). However, like the full-length HKα2 subunit (16, 18, 19), HKα2b is insensitive to high concentrations of Sch 28080. Moreover, the approximate IC_{50} for ouabain inhibition (400–800 μM in the presence of external 1 mM K^+) of the HKα2b/HKβ_g pump reported here is comparable to values reported for the full-length HKα2 subunit expressed in heterologous systems. Codina *et al.* (16) reported an IC_{50} of 400–600 μM in the presence of external 1 mM K^+ for HKα2 pumps expressed in *Xenopus* oocytes, and Cougnon *et al.* (18) reported K_i values for ouabain of ~70 and ~970 μM in the presence of external 0.2 and 5 mM K^+, respectively, for HKα2 pumps expressed in HEK 293 cells. Clearly, heterologous expression and detailed functional analysis of the two isoforms in a common host cell will be needed to distinguish subtle differences.

These considerations take on added meaning when viewed in the context of recent functional studies in kidney and colon. *In vitro* studies have identified at least three different K^+-ATPase activities that are distinguished by their kinetic and pharmacological properties in rat kidney (51, 52). One activity (type I) is K^+-, but not Na^+-dependent, ouabain-resistant, Sch 28080-sensitive, and expressed in collecting ducts. A second activity (type II) is K^+-, but not Na^+-dependent, Sch 28080- and ouabain-sensitive, and expressed basally in proximal tubules and the thick ascending limbs (52). This activity is virtually abolished during chronic K^+ depletion. A third activity (type III) is activated by either Na^+ or K^+, exhibits higher sensitivities to ouabain and to Sch 28080 than type II, and a lower sensitivity to Sch 28080 than type I. This activity is not expressed basally but is specifically up-regulated in cortical collecting ducts and OMCDs with chronic hypokalemia (52). Similarly, both ouabain-sensitive and insensitive K^+-ATPase activities have been identified in the apical membranes of colonocytes from the distal colon (10), yet only HKα2 mRNA (5, 8) and protein (8) have been identified in these cells. These collective data have led us to postulate that a yet-to-be discovered K^+-ATPase isoform may be operative in the renal collecting duct and colon (8, 19, 51). It is possible that functional differences in the HKα2 protein variants may account for these puzzling data.

In addition to the generation of protein isoforms differing at the N terminus, the use of alternative promoters in the HKα2 gene would be expected to afford considerable versatility in controlling its expression. Alternative promoter usage in other genes has been shown to allow for expression of isoforms exhibiting differences in the degree and timing of transcription initiation, mRNA turnover, translational efficiency, tissue specificity, and responses to signal transduction pathways (53). The HKα2 gene appears to be the first example of a P-type ATPase to employ alternative promoters and mRNA splicing to generate structural and regulatory diversity. This mechanism, then, adds to the known complexity of X^+-K^+-ATPase regulation, which includes controls on transcription, translational efficiency, subunit assembly, and various post-translational modifications. It may also provide an explanation for the well documented differential expression of the HKα2 gene in kidney and distal colon under various experimental conditions. For

example, Jaisser and co-workers (5) showed that chronic K^+ deprivation did not alter, adrenalectomy reduced, and dexamethasone supplementation of adrenalectomized rats restored steady-state HKα2 mRNA levels in distal colon. In contrast, chronic K^+ deprivation enhanced HKα2 mRNA expression in the OMCD, whereas adrenalectomy did not alter HKα2 gene expression. We (6) and others (7) have shown similar effects of K^+ deprivation on HKα2 mRNA levels in the OMCD. The probes used in all these studies would be expected to hybridize to both HKα2 variants. Similarly, Sangan and colleagues (8) showed that chronic dietary Na^+ depletion (presumed to promote secondary hyperaldosteronism), but not chronic K^+ depletion, enhanced HKα2 mRNA and protein levels in distal colon. Conversely, chronic K^+ depletion promoted HKα2 protein but not mRNA expression in outer medulla, whereas Na^+ depletion did not affect renal expression of this gene product. Fortuitously, the antibody (termed M-1) used in this and an earlier (19) study was raised against a fusion protein produced from the first 109 amino acids of the HKα2a sequence. Thus, this antibody would be expected to be specific for HKα2a, and it would not detect HKα2b. M-1 immunoreactivity was identified in the apical membranes of principal cells of the K^+-deprived OMCD (8) and of surface cells in rat distal colon (19). Since the consensus of *in situ* hybridization studies (5, 6) with probes common to the two HKα2 variants indicated that HKα2 mRNA is primarily expressed in OMCD intercalated cells, it is reasonable to hypothesize that the two isoforms are expressed in different cell types of the rat OMCD during K^+ depletion. The sequence information presented here should facilitate future studies to define the molecular mechanisms controlling the differential and cell type-specific expression of these isoforms.

Finally, although it has been hypothesized that HKα2 and ATP1AL1 represent species variants of the same protein, the novel structural organization and regulatory mechanisms for HKα2 transcription described here add to the growing list of differences that suggest that these proteins represent distinct protein isoforms. These distinguishing features include the contrasting tissue distributions (20), differences in pharmacological profile (16, 18, 19, 21, 22), and greater degree of sequence divergence when compared with the interspecies differences of the other human and rat X^+-K^+-ATPase α subunit isoforms. As additional structure-function and structure-regulation correlations for these genes are identified, their evolutionary relationship should come into clearer focus.

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