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Segmental localization of mRNAs encoding Na^+-K^+-ATPase α- and β-subunit isoforms in rat kidney using RT-PCR

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Segmental localization of mRNAs encoding Na^+-K^+-ATPase α- and β-subunit isoforms in rat kidney using RT-PCR. To characterize the expression of genes encoding the α- and β-subunit isoforms of the Na^+-K^+-ATPase in rat kidney, we used reverse transcription (RT)-PCR of microdissected renal structures combined with quantitation of subunit isoform mRNAs in the major renal parenchymal zones. Transcripts for $\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, and $\beta2$ subunit isoforms were detected by RT-PCR in microdissected glomeruli, proximal convoluted tubules, medullary thick ascending limbs of Henle, cortical and inner medullary collecting ducts. The truncated $\alpha1$ ($\alpha1$-T) isoform was also amplified from cortex, outer and inner medulla and isolated glomeruli, but it was not detected in these nephron segments. The DNA sequence of the renal $\alpha1$-T PCR product was identical to that of the cDNA previously cloned from aortic smooth muscle cells. RNA dot-blot analysis indicated that the $\alpha1$, $\alpha2$, and $\alpha3$ isoforms contributed ~70%, ~20%, and ~10%, respectively, of the total α isoform mRNA in each parenchymal zone. RNase protection assays determined that the $\beta1$ and $\beta2$ isoforms accounted for ~95% and ~5%, respectively, of the β isoform mRNA in each zone. These data provide definitive evidence for the differential expression of mRNAs encoding all the α and β isoforms in the renal parenchyma, and for the coexpression of these isoforms in the nephron segments examined. The results suggest the potential expression of up to eight different Na^+-K^+-ATPase isoenzymes in the kidney, and for multiple molecular levels of regulation of renal Na^+-K^+-ATPase expression.

The Na^+-K^+-ATPase is an oligomeric membrane protein responsible for the primary active transport of Na^+ and K^+ in all animal cells. By maintaining or restoring the normal distribution of these ions across the plasma membrane, the Na^+-K^+-ATPase plays a central role in the regulation of membrane potential, cell ion content, and cell volume. In renal tubular epithelial cells, this enzyme provides the principal driving force for net sodium reabsorption, as well as for the secondary active transport of other ions and organic solutes [1]. Structurally, the enzyme is comprised of two protein subunits linked by noncovalent bonds: the ~100 kD α subunit is responsible for ATP hydrolysis, cation transport, and cardiac glycoside (that is, ouabain) binding, whereas the ~40 to 50 kD glycoprotein β subunit appears to play a role in directing the α subunit to the plasma membrane [2], in K^+ activation of the holoenzyme [3], and perhaps in cellular adhesion [4]. The two subunits are encoded by multigene families, and appear to be differentially expressed among tissues and during development [5–11]. In rat, cDNAs encoding three α- [6, 12] and two β-subunit [4, 7, 8] isoforms of the Na^+-K^+-ATPase have been cloned and sequenced, and a truncated $\alpha1$ isoform, termed $\alpha1$-T, has recently been identified in aortic smooth muscle cells and canine kidney [13]. The $\alpha1$-T isoform appears to arise by alternative RNA processing of the $\alpha1$ gene and encodes a protein of 581 amino acids whose functional properties, ability to assemble with the β subunit, and precise renal expression are unknown. The $\alpha1$ subunit is expressed ubiquitously among tissues and is thought to play a "housekeeping" role in maintaining Na^+ and K^+ gradients, whereas the $\alpha2$ and $\alpha3$ subunits differ in their tissue expression [reviewed in 14, 15], regulation, and in affinity for Na^+ and K^+ [16, 17]. The $\alpha2$ subunit has been identified principally in neural and muscle tissue, and the $\alpha3$ isoform has been detected primarily in neural tissues.

Because the different Na^+-K^+-ATPase isoenzymes are subject to unique regulatory controls and appear to possess distinct functional properties [15–17], it has been proposed that differences in their expression or regulation might account for the known functional diversity of the Na^+-K^+-ATPase along the nephron. Biochemical studies have demonstrated differences in the affinities of the enzyme for ouabain and Na^+ [18–20] among different nephron segments. Recent studies of the rat cortical collecting duct (CCD), for example, distinguished two distinct populations of Na^+-K^+-ATPase molecules based on their affinities for ouabain [20]. The authors further observed that the low-affinity Na^+-K^+-ATPase population of the CCD was differentially stimulated in rats with puromycin-induced nephrotic syndrome. Since the rat α isoforms possess very different sensitivities to ouabain, these results implied that $\alpha1$ (low ouabain affinity) together with $\alpha2$ and/or $\alpha3$ (high ouabain affinity) isozymes are coexpressed in this nephron segment, and that they respond differently to the same pathophysiologic stimulus. In addition, Barlet-Bas et al [21] reported that an antibody specific for the $\alpha3$ isoform inhibited >80% of Na^+-K^+-ATPase activity in the rabbit CCD, whereas an $\alpha1$-specific antibody had no effect on enzymatic activity in this segment.

Unfortunately, analysis of the specific α- and β-subunit isoform genes in the kidney has been complicated by both the molecular variety of the Na^+-K^+-ATPase isoforms and the complex structural heterogeneity of this organ. In general, previous studies of Na^+-K^+-ATPase isoform gene expression in the kidney have

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yielded inconsistent results and have been limited by their inability to determine unambiguously the molecular identity and intrarenal location of the Na^+-K^+-ATPase isoforms under question. Recently we reported that the major α and β isoforms are coexpressed in specific segments of the rat nephron as detected by *in situ* hybridization with isoform-specific riboprobes [22]. While this study provided a useful map of the cellular distribution of the various Na^+-K^+-ATPase isoforms, it did not characterize the relative abundance of the subunit isoform mRNAs. Moreover, even though the riboprobes used in that study shared less than 70% identity with one another, cross-hybridization of the probes with homologous isoforms was difficult to exclude conclusively by this method. Therefore, in the present study we have used RNA dot-blot analysis and RNase protection assays to characterize the steady-state renal expression of the various α and β subunit isoform mRNAs, and reverse transcription-PCR (RT-PCR) combined with nephron microdissection and nucleotide sequence analysis to determine definitively the segmental location of the specific mRNAs encoding the Na^+-K^+-ATPase isoenzymes in the normal rat kidney. Our results demonstrate that $\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, and $\beta2$ subunit mRNAs are coexpressed in glomeruli and defined segments along the nephron. Moreover, we report that $\alpha1$-T mRNA is expressed in each parenchymal zone and isolated glomeruli, but not in several tubule segments, and that it is structurally identical to that cloned from vascular smooth muscle.

Methods

Animals

Male Sprague Dawley rats (150 to 200 g body wt were allowed free access to tap water and standard chow.

Macrodissection and microdissection

For macrodissection of cortex, outer and inner medulla, rats were anesthetized with sodium pentobarbital (10 mg/kg i.p.), and the kidneys were perfused free of blood via the abdominal aorta with an ice-cold buffer (Buffer A) containing (in mM) 135 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.0 Na_2HPO_4, 1.2 Na_2SO_4, 2.0 $CaCl_2$, 5.5 glucose, 5.0 HEPES, pH 7.4. The kidneys were excised, and the three zones isolated with dissection scissors. For microdissection of nephron segments, we used the protocol described by Moriyama et al [23] with a few modifications. Following anesthesia of the animal, the kidneys were infused via the abdominal aorta with ice-cold Buffer A containing 1.5 mg/ml collagenase (Type I, Sigma Chemical, St. Louis, Missouri, USA) and 0.38 mg/ml Pronase (Sigma) that was bubbled with 100% O_2. The kidneys were excised, and thin coronal slices were cut and incubated at 37°C for 20 to 25 minutes for inner medullary collecting ducts (IMCD), or 30 to 40 minutes for glomeruli, proximal convoluted tubules (PCT), medullary thick ascending limbs of Henle (mTAL), and CCDs, in Buffer A containing 1.5 mg/ml collagenase but only 0.19 mg/ml Pronase. Microdissection was carried out at 4°C in Buffer A without the enzymes. In contrast to other investigators [22], we did not include vanadyl ribonucleoside complex in the dissection medium, because we found that it increased the difficulty of dissection (by rendering the medium more opaque) without significantly improving the quantity or apparent quality of the RNA isolated from the tubules. We had roughly equivalent success in amplifying the various Na^+-K^+-ATPase isoform cDNAs reverse transcribed from RNA harvested when vanadyl ribonucleoside complex was omitted from the dissection medium as when it was included. However, this result may not be the case for all target genes or for other microdissected renal structures. The tubule structures were dissected with fine steel forceps, their lengths measured, and then they were transferred with a micro-glass tube precoated with 1% bovine serum albumin (RNase-free, Sigma) to a wash dish in which they were quickly rinsed free of debris. After two washes, 4 to 6 tubules (each ~2 mm in length) or 8 to 10 glomeruli were then pipetted directly into a microcentrifuge tube, flash frozen in a dry ice/ethanol bath, and stored at −80°C until the time of RNA isolation. As a control for possible contamination, 10 μl of the final wash buffer was carried through the RNA isolation, reverse transcription, and PCR steps.

RNA isolation, cDNA synthesis, and PCR

Total RNA was extracted from the heart, brain, macrodissected cortex, outer medulla, or inner medulla, or from pooled tubules or glomeruli using a commercially available kit, RNAzol II (TEL-TEST, Inc., Friendswood, Texas, USA). For the glomeruli and tubule segments, we scaled down the procedure to smaller volumes. The final RNA pellets from heart, brain, and renal cortex, outer, and inner medulla were resuspended in 100 μl of diethylpyrocarbonate (DEPC)-treated water and quantified by absorbance at 260 nm. The RNA pellets isolated from the glomeruli and tubules were resuspended in DEPC-treated water such that 5 μl of RNA corresponded to ~1 mm tubule or ~2 glomeruli. First strand cDNA was synthesized from 1 μg of total RNA harvested from each of the three macrodissected zones or from 5 μl of the microdissection RNA sample, using oligo-$(dT)_{17}$ primer, 200 U MMLV reverse transcriptase (Gibco-BRL, Gaithersburg, Maryland, USA), and the buffer supplied by the manufacturer in a 20 μl reaction volume for one hour at 37°C. The reaction was stopped by incubation at 99°C for five minutes, and the cDNA was stored at −80°C until used.

Isoform-specific primers (Table 1) were selected by comparative nucleotide sequence analysis of published cDNA sequences [7, 8, 12, 13] and with the aid of the Oligo 4.0 Primer Design Software (National Biosciences, Inc., Plymouth, Minnesota, USA). With the exception of the β_2 primers, each of the primers contained recognition sequences for restriction endonucleases to facilitate directional subcloning. Given the considerable structural homology of the various α subunit isoforms, we exploited the sequence differences in the 3′ termini of the isoforms to lend specificity to our primers, and to frame regions of structural diversity for use as isoform-specific probes in blot and *in situ* hybridization studies [22]. The reverse primer of the $\alpha1$ subunit was located 223 bases into the 3′ untranslated region, whereas the $\alpha2$ primer was located 40 bases into the 3′ untranslated region. For amplification of the $\alpha1$-T transcript, the forward primer began with the third nucleotide of Cys^{459} of $\alpha1$ located on exon 11, and the reverse primer began at the termination codon of the $\alpha1$-T open reading frame located in intron 12.

Although the genomic sequences for the isoforms in the rat are unknown, the exon-intron structure of the α subunit genes is conserved between widely divergent species (chickens [24] and humans [25–28]). Assuming similar genomic organization in the rat, the α isoform primer pairs would span at least one intron. Likewise, if one assumes similar exon-intron arrangement between rat and the cloned mouse β subunit genes [29, 30], the β

Table 1. Oligonucleotide primers used for RT-PCR of Na^+-K^+-ATPase subunit isoforms

Isoform	Primer sequence	Nucleotides of cDNA
α1		
Sense	5′ CCGGAATTCTGCCTTCCCCTACTCCCTTCTCATC 3′	3207-3529
Antisense	5′ TGCTCTAGACTTCCCCGCTGTCGTCCCCGTCCAC 3′	
α1T		
Sense	5′ CGCCTGCAGATCGAGGTCTGCTGTGGCT 3′	1615-1897
Antisense	5′ GTCGACGAATTCTACGTTAAGAGGAACAC 3′	+ intron
α2		
Sense	5′ CCGGAATTCCGGCTTCTTCACCTACTTTGTAATA 3′	2681-3192
Antisense	5′ TGCTCTAGAATCTCCCCTGTTCTTCCTTTTGTCG 3′	
α3		
Sense	5′ CCGGAATTCTCGCCTACACTCTGCACCAGCAACAT 3′	2440-2742
Antisense	5′ GCGGGATCCCATTTTCTGCCAGGATGACAAAGTA 3′	
β1		
Sense	5′ GTCGAATTCCCTTCCGTCCTAATGACCCCAAGA 3′	725-943
Antisense	5′ GCGGGATCCGACCAGAGCAGTTCCCCAGCCAGTC 3′	
β2		
Sense	5′ TTGGGAAGAAAGATGAAGAT 3′	1063-1366
Antisense	5′ AGGAGGGTATGGGTGAGAGG 3′	

cDNA sequences for the various subunit isoforms were obtained from published work: α1, α2, and α3 [12], α1-T [13], β1 [8], and β2 [7].

subunit primers would also span at least one intron. PCR was performed in a Perkin Elmer 4800 Thermal Cycler (Perkin Elmer-Cetus, Norwalk, Connecticut, USA) on identical 2 to 4 μl aliquots of the first strand cDNA with the GeneAmp kit (Perkin Elmer-Cetus). The PCR mixture contained 25 to 50 pmol of each primer, 200 μM of each dNTP, 2.5 units of Ampli*Taq* polymerase, 10 mM dithiothreitol, 10 μM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 to 2.5 mM $MgCl_2$ in a 50 to 100 μl volume. The concentrations of primers and $MgCl_2$ were optimized for each primer pair. For the α1, α3, β1, and β2 isoforms, amplification was performed as follows: 95°C × two minutes (initial denaturation), 35 cycles of 95°C × one minute, 55°C × one minute, and 72°C × two minutes, followed by a final extension at 72°C × seven minutes. For the α2 primers, the amplification consisted of 95°C × one minute, 37°C × one minute, and 72°C × two minutes for 3 cycles, followed by 35 cycles of 95°C × one minute, 55°C × one minute, and 72°C × two minutes, and a final extension at 72°C × seven minutes. To control for genomic DNA contamination of the cDNA samples, the original RNA was directly amplified with each set of primers without reverse transcription. Moreover, PCR of the common "master mix" of reagents and primers was performed in the absence of template DNA or RNA to exclude the possibility of sample contamination.

Analysis, cloning, and sequencing of PCR products

Our previous molecular cloning and nucleotide sequencing data [22] demonstrated the authenticity of the α1, α2, α3, and β2 products amplified with the respective primers from renal cortex. To verify the molecular identities of the β1 and α1-T PCR products, 10 μl of the PCR products amplified from renal cortex were size-fractionated by electrophoresis through a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide in Tris borate/EDTA buffer. Ethidium bromide-stained product bands of expected size were visualized under ultraviolet (UV) light, excised from the gel, and the DNA purified using the PREP-A-GENE kit (Bio-Rad Laboratories, Richmond, California, USA). The purified DNA was then cut with the respective restriction endonucleases, subcloned into pBluescript KS+ (Stratagene, La Jolla, California, USA), and transformed into DH5α competent cells (Gibco-BRL). Recombinant plasmid DNA was purified by the alkaline lysis method and used as double-stranded templates for sequencing with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio, USA). In subsequent experiments, the size-fractionated PCR products amplified from the microdissected renal structures with the α1, α2, α3, β1, and β2 primers were transferred to nylon membranes and hybridized at high stringency to digoxigenin-labeled cDNA probes specific for each isoform PCR product. The probes were generated by PCR amplification of the cloned cDNA fragments of the different isoforms in the presence of digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Indiana, USA) using the isoform-specific primers employed in the original amplification [31]. The blots were washed to a final stringency of 0.1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 0.1% SDS at 68°C. Immunological detection of DNA hybrids was carried out using alkaline phosphatase-conjugated anti-digoxigenin antibody and 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride as described in the technical bulletin accompanying the Genius DNA Labeling and Detection Kit (Boehringer Mannheim). The blots were photocopied onto transparency sheets and then photographed. A product migrating at the predicted size that hybridized under high stringency conditions with the cloned and sequenced cDNA probe from renal cortex was interpreted as a positive result.

cRNA probe preparation

The cloned α1 and β2 PCR products [21] (Table 1) were used to generate isoform-specific riboprobes. Isoform-specific cDNA fragments for the α2 (nucleotides 121-502), α3 (nucleotides 50-330), and β1 (nucleotides 913-1184) subunits were provided by Dr. Jerry Lingrel, University of Cincinnati. The isoform specificity of these probes has been well established [9, 11, 12]. The β1 fragment was cloned into the *Hind*III and *Pst*I sites of the plasmid vector pIBI30 (IBI), whereas the α3 cDNA fragment was subcloned into the *Pst*I and *Sma*I sites of pBluescript KS+. To increase the isoform specificity of the α2 probe further (from ~68% to < 50% identity with the corresponding regions of the α1

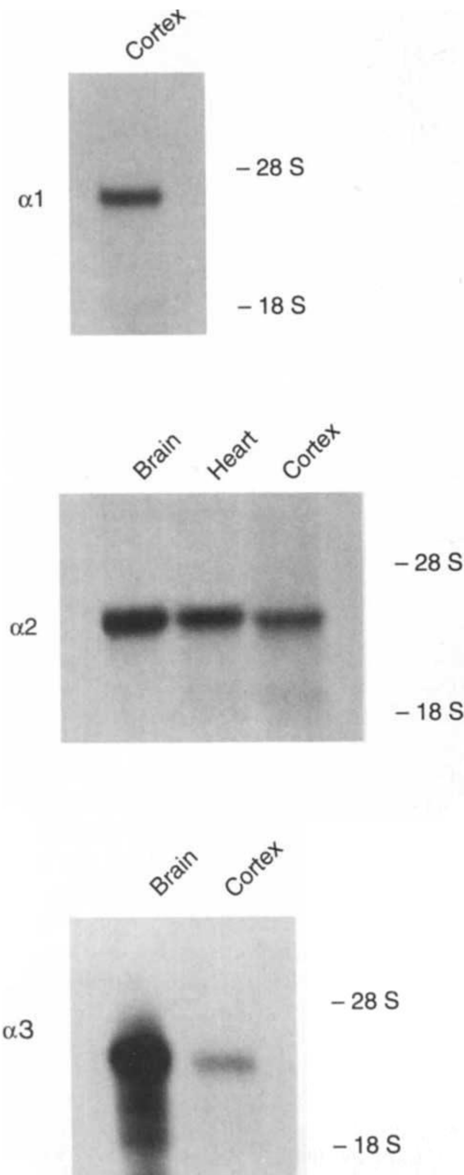

Fig. 1. *Northern analysis of Na^+-K^+-ATPase α isoform mRNAs.* Total RNA from rat kidney cortex (10 μg for α1, 20 μg for α2 and α3), brain (10 μg), and heart (10 μg) were electrophoresed and blotted onto nylon membranes. Separate filters were hybridized with ^{32}P-labeled cRNA probes specific for each α subunit isoform as described in **Methods**. An autoradiogram for each probe is shown. Migration positions of 28S and 18S RNA bands are shown at the right.

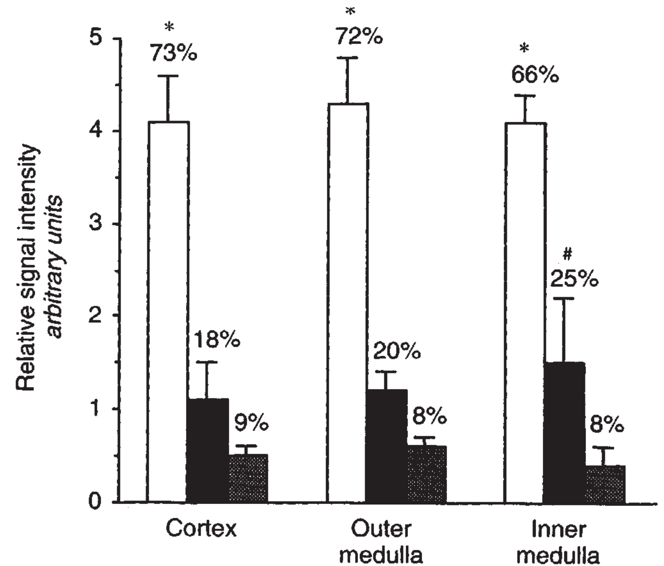

Fig. 2. *Relative abundance of α subunits isoforms of the Na^+-K^+-ATPase in the renal parenchyma.* Dot blots were prepared by hybridization of total RNA harvested from the indicated renal zones with ^{32}P-labeled antisense RNAs for each α subunit isoform. The relative signal intensities of the dots were measured by scanning densitometry of autoradiograms, and the data were corrected for size differences of the probes. The histogram presents the mean ± SEM of the corrected data obtained from three experiments from separate rats. The percentages above each bar indicate the percentage of the total mRNA abundance contributed by the mean values of each α isoform in each zone. The sum of the α1, α2, and α3 isoform mRNA levels was assumed to represent 100% of total, full-length α isoform mRNA. $*P < 0.05$ vs. α2 and α3 values in each zone; $\#P < 0.05$ vs. cortex α3 level.

and α3 subunits), a 109 bp *Sac*I-*Sac*I segment (nucleotides 183-292) of the α2 cDNA was subcloned into the *Sac*I site of pBluescript KS+ for use as a probe. Using the University of Wisconsin Genetics Computer Group Sequence Analysis GAP program to calculate the degree of identity of the α isoform and the β isoform probes when optimally aligned with their homologues, the α isoform probes were < 55% identical, whereas the β isoform probes were < 50% identical. Moreover the probes showed no significant homology with any other ion motive ATPase in the current GENBANK database.

The recombinant plasmids were linearized with appropriate restriction endonucleases to allow synthesis of cRNAs as run-off transcripts with T7 RNA polymerase. One microgram of linearized recombinant plasmid was incubated for two hours at 37°C in a solution containing transcription buffer, 20 U of human placental RNase inhibitor (Boehringer Mannheim), 10 mM dithiothreitol, GTP, ATP, CTP (1 mM each), 0.65 mM unlabeled UTP, 0.35 mM [α-^{32}P]-UTP (800 Ci/mmol; Amersham Corp.), and 50 U of T7 RNA polymerase (Ambion). The reaction was terminated by digestion of the DNA template with 10 U RNase-free DNase I (Sigma), and the labeled riboprobes were precipitated in ethanol.

Northern and dot-blot analyses

Total RNA (10 to 20 μg) isolated from renal cortex, brain, or heart was electrophoresed through 1% agarose-formaldehyde gels, transferred to nylon membranes (Boehringer-Mannheim), and immobilized by UV cross-linking. The membranes were prehybridized for a minimum of two hours at 52°C in 50% formamide, 5X SSC, 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrolidine, 0.1% bovine serum albumin), 0.2% SDS, 200 μg/ml denatured salmon sperm DNA, and 200 μg/ml yeast tRNA. For hybridization, 5×10^6 cpm/ml ^{32}P-labeled cRNAs for α1, α2, α3, β1, and β2 isoforms of the Na^+-K^+-ATPase were added to separate membranes, and hybridized at 52°C for 16 hours. The filters were washed three times at high stringency in 0.1X SSC-0.1% SDS at 65°C for 30 minutes each wash, and exposed to X-ray film using an intensifying screen for 4 to 24 hours at −70°C.

For dot-blot analysis, serial dilutions (0.15, 0.3, 0.6, 1.25, 2.5,

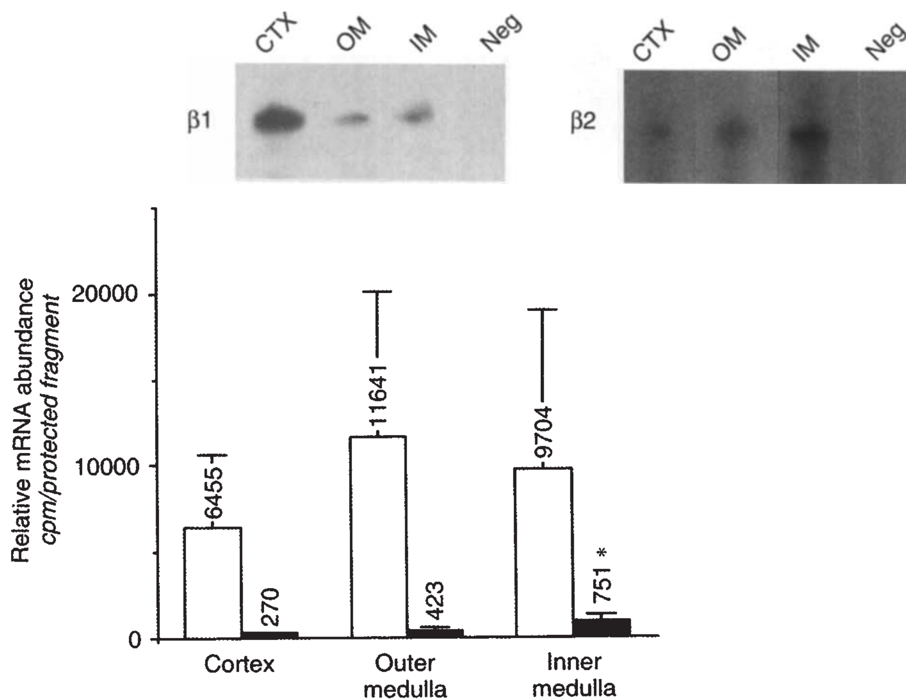

Fig. 3. *Ribonuclease protection of kidney RNA using radiolabeled riboprobes specific for the β1 (□) and β2 (■) subunits of the Na^+-K^+-ATPase.* Representative autoradiograms (insets) showing that the isoform-specific riboprobes protected bands of the anticipated size in each parenchyma zone (insets), but not when hybridized to yeast RNA (Neg). The protected bands were excised from the gels, counted for β emissions, and the compiled data ($N = 3$) were plotted for each zone. *$P < 0.05$ vs. cortex β2. Abbreviations are: CTX, cortex; OM, outer medulla; IM, inner medulla.

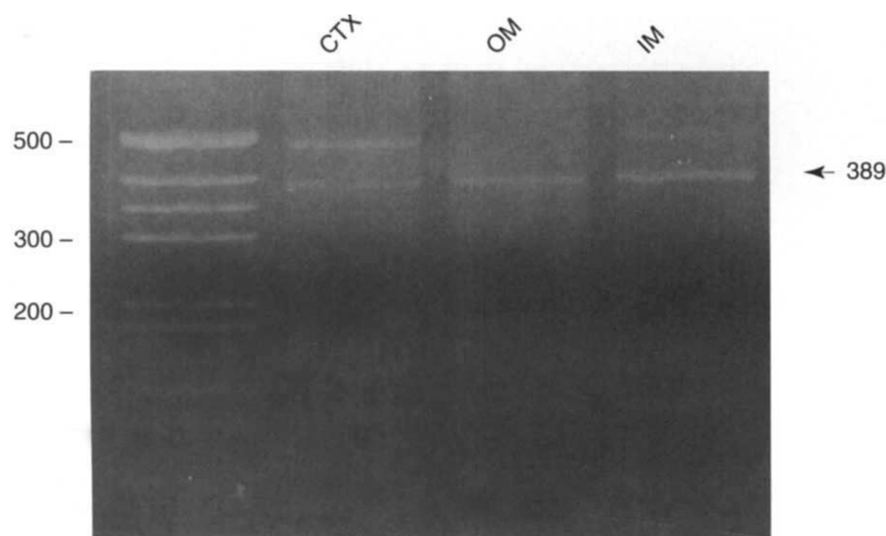

Fig. 4. *Amplification and detection of mRNA encoding α1-T isoform of the Na^+-K^+-ATPase subunit in rat kidney by RT-PCR.* Representative ethidium bromide-stained 1.5% agarose gel showing PCR products obtained by amplification of cDNA from the indicated structures with the α1-T primers. Molecular size standards were the 1 kb DNA ladder from GIBCO-BRL. Abbreviations are in Figure 3.

and 5 μg) of total RNA from the renal cortex, outer and inner medulla were denatured in 6X SSC, 7% formaldehyde, pH 4.7, for 15 minutes at 60°C. The denatured RNA was transferred by vacuum blotting to nylon membranes (Boehringer Mannheim) using a dot-blot apparatus (GIBCO-BRL), and immobilized on the membranes by UV cross linking. Prehybridization, hybridization, and washing were performed under identical conditions as in the Northern analysis, except that 3.6×10^6 cpm/ml of the ^{32}P-labeled cRNAs for the α1, α2, and α3 subunits were used. The blots were exposed to X-ray film using an intensifying screen for 4 to 24 hours at −70°C, and the film was then developed and scanned for densitometry. All of the steps in this analysis were performed in parallel for the different α isoforms.

RNase protection assay

Relative quantitation of β1- and β2-subunit mRNAs in the renal parenchymal zones by RNase protection assay was performed using the RPAII Kit (Ambion) according to the manufacturer's instructions. Ten to 25 μg of total RNA were hybridized with 4×10^4 cpm of the probe in 20 μl of hybridization solution (80% formamide, 0.1 M sodium citrate, pH 6.4, 0.3 M sodium acetate, pH 6.4, 1 mM EDTA) at 42°C. After digestion with

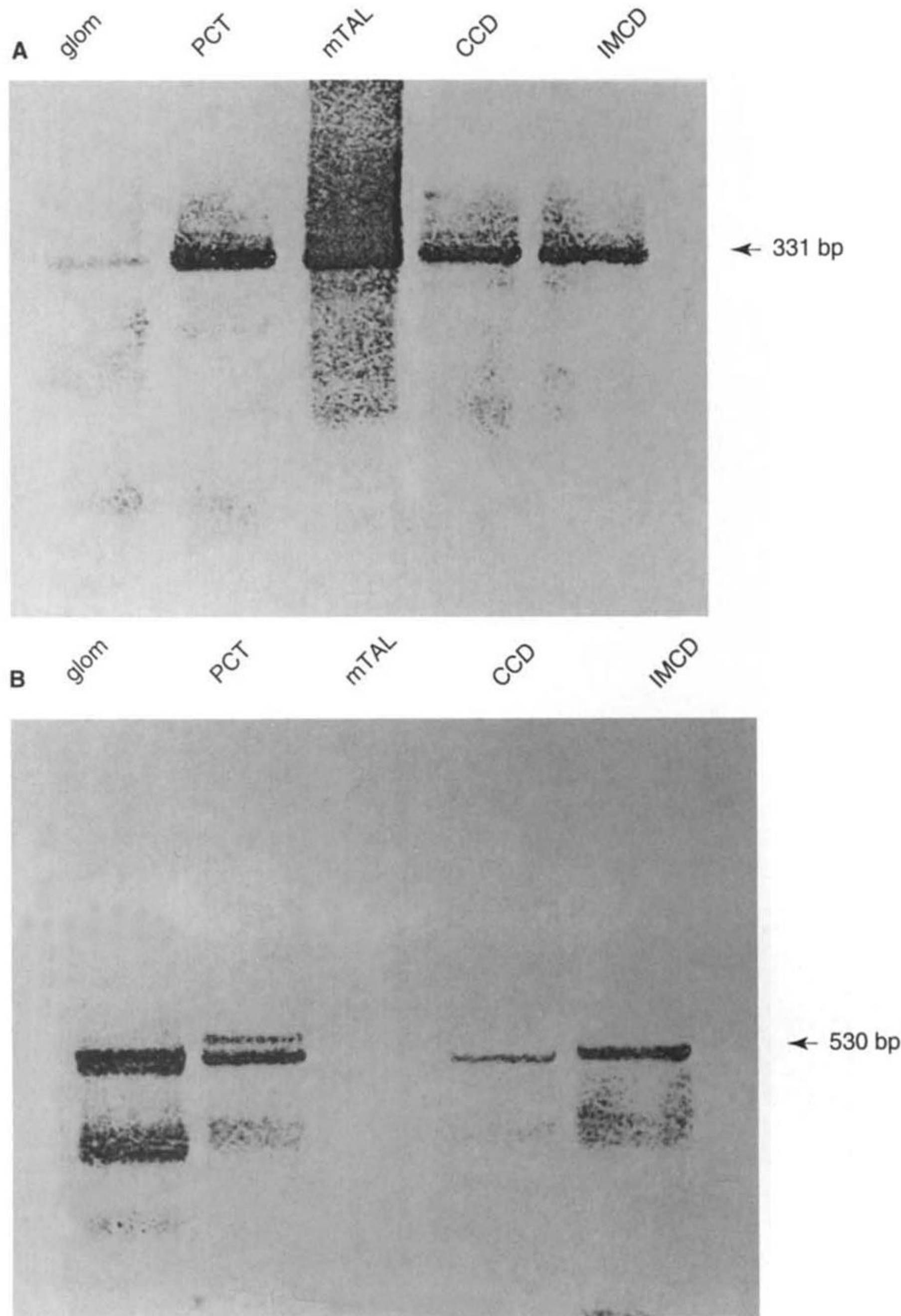

Fig. 5. *Expression of Na^+-K^+-ATPase α subunit isoform mRNAs in microdissected structures from rat kidney.* (A–C) Southern blots of size-fractionated PCR products amplified from the indicated microdissected segments with α isoform-specific primers: (**A**) $\alpha 1$, (**B**) $\alpha 2$, and (**C**) $\alpha 3$. The blots were probed with digoxigenin-labeled cDNAs specific for each α isoform. (**C**) Ethidium bromide-stained agarose gel of RT-PCR products amplified with the $\alpha 1$-T primers. Molecular weight standards were the 1 kb ladder from GIBCO-BRL. Glom is glomerulus; other abbreviations are in text.

RNases A and T1, the samples were electrophoresed on 6% polyacrylamide/7 M urea gels. The areas corresponding to the protected bands were identified by autoradiography, excised from the gel, and their incorporated radioactivity was measured by liquid scintillation counting. As a negative control, the probes were hybridized with 10 μg of yeast RNA.

Quantitation and statistical analysis

Multiple exposures of autoradiograms were analyzed to ensure that signals were within the linear range of the film. Dots on autoradiograms were scanned with a Micro-Scan 1000 Gel Analyzer (General Image Corp., Gainesville, Florida, USA), and the peaks were integrated with TRI ID Gel Analysis System (Technology Resources, Inc., Nashville, Tennessee, USA) software. The signal intensity increased in a linear fashion with increasing amounts of total RNA (0.15 to 1.25 μg) from each zone and with each probe. Dots within this linear range were analyzed for comparative quantitation. After correction for size and specific activities of the probes, the means ± SEM were calculated. Where appropriate the data were analyzed for significance by the Student's t-test for unpaired data and accepted at $P < 0.05$.

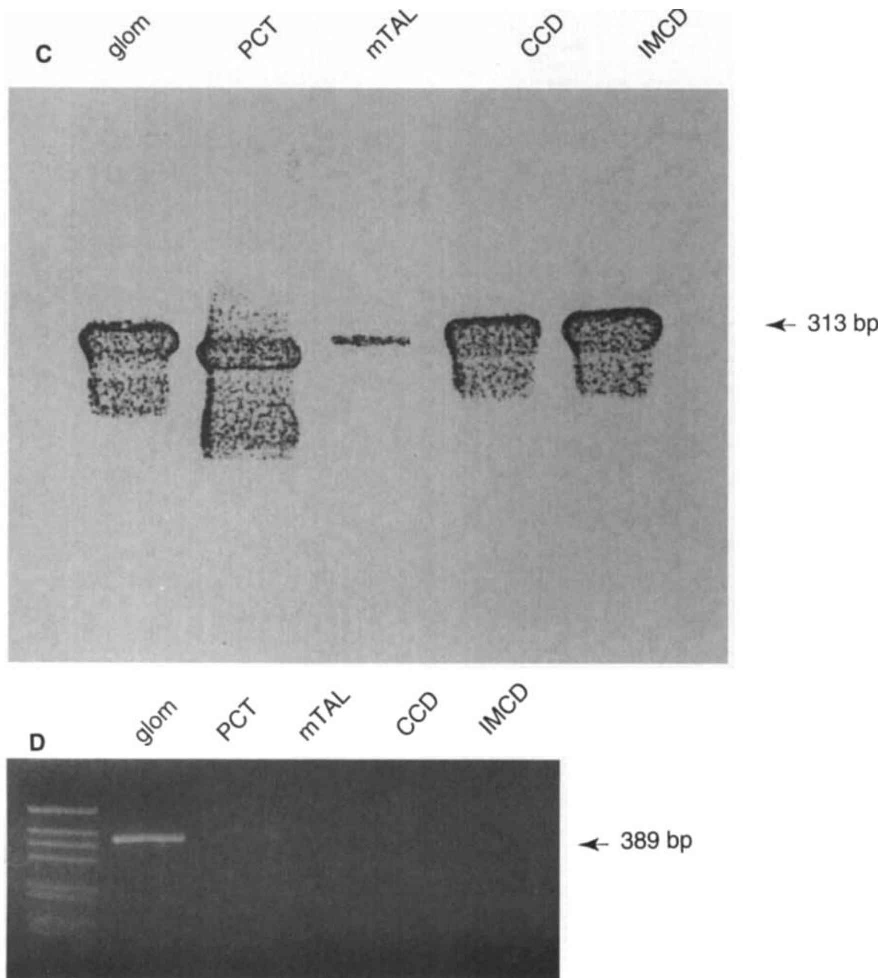

Fig. 5. *Continued*

Results

Analysis of Na-K-ATPase mRNA in zones of kidney parenchyma

Previous studies of steady-state mRNA abundance of the Na^+-K^+-ATPase subunit isoforms [5–11] were limited to analysis of RNA isolated from whole kidney, and did not discriminate the zonal distribution of isoform transcripts in this organ. Since differences in the transcript abundance of specific Na^+-K^+-ATPase subunit isoforms in distinct regions of the kidney could be obscured by analysis of whole kidney RNA, we prepared isoform-specific cRNA probes and used them to analyze dot-blots of total RNA prepared from the cortex, outer and inner medulla of the normal rat kidney. To demonstrate that the probes identified transcripts of the size expected for the α (Fig. 1) and β subunit isoforms, Northern hybridizations were performed on total RNA prepared from kidney cortex, heart, and brain. The latter tissues were used as positive controls, since the $\alpha2$ isoform is prominently expressed in heart and brain, and the $\alpha3$ isoform is abundant in brain [5–11]. The $\alpha1$ riboprobe hybridized to an ~3.7 kB mRNA species in renal cortex, whereas the $\alpha2$ probe detected ~3.4 kB transcripts in heart, brain, and kidney cortex. The $\alpha3$ probe hybridized intensely to an ~3.8 kB mRNA in brain and, more weakly to an identical transcript in kidney cortex. Since twice as much renal cortex mRNA as heart and brain mRNA was analyzed, the levels of $\alpha2$ and $\alpha3$ mRNAs in the kidney cortex appeared to be much less than in these other tissues. Northern hybridization (not shown) with the $\beta1$ riboprobe identified an ~2.4 kB transcript in each renal zone, whereas $\beta2$ transcripts (~2.2 kB) were observed only after prolonged (> 48 hr) exposures of the film. Taken together, these data indicated that the isoform-specific riboprobes hybridized to the appropriate mRNA species.

Dot-blot analysis was then performed to assess the relative, steady-state mRNA levels of the α isoforms in the three parenchymal zones (Fig. 2). Scanning densitometry of the dot-blot autoradiograms revealed that $\alpha1$ was the predominant mRNA species throughout the kidney, and that it was expressed at comparable levels in all three zones. The relative levels of $\alpha1$ mRNA were ~4- and ~8 times the amount of $\alpha2$ and $\alpha3$ transcripts, respectively, in each zone. For each α isoform, the relative mRNA levels appeared to be approximately the same from zone to zone. In each experiment, the approximate level of $\alpha2$ mRNA was greater than that of the $\alpha3$ isoform in each zone, and the mean relative level of $\alpha2$ mRNA in the inner medulla was significantly greater than that of $\alpha3$ mRNA expressed in cortex.

Given the difficulty in detecting the $\beta2$ mRNA by Northern analysis, we used RNase protection assays to compare the relative

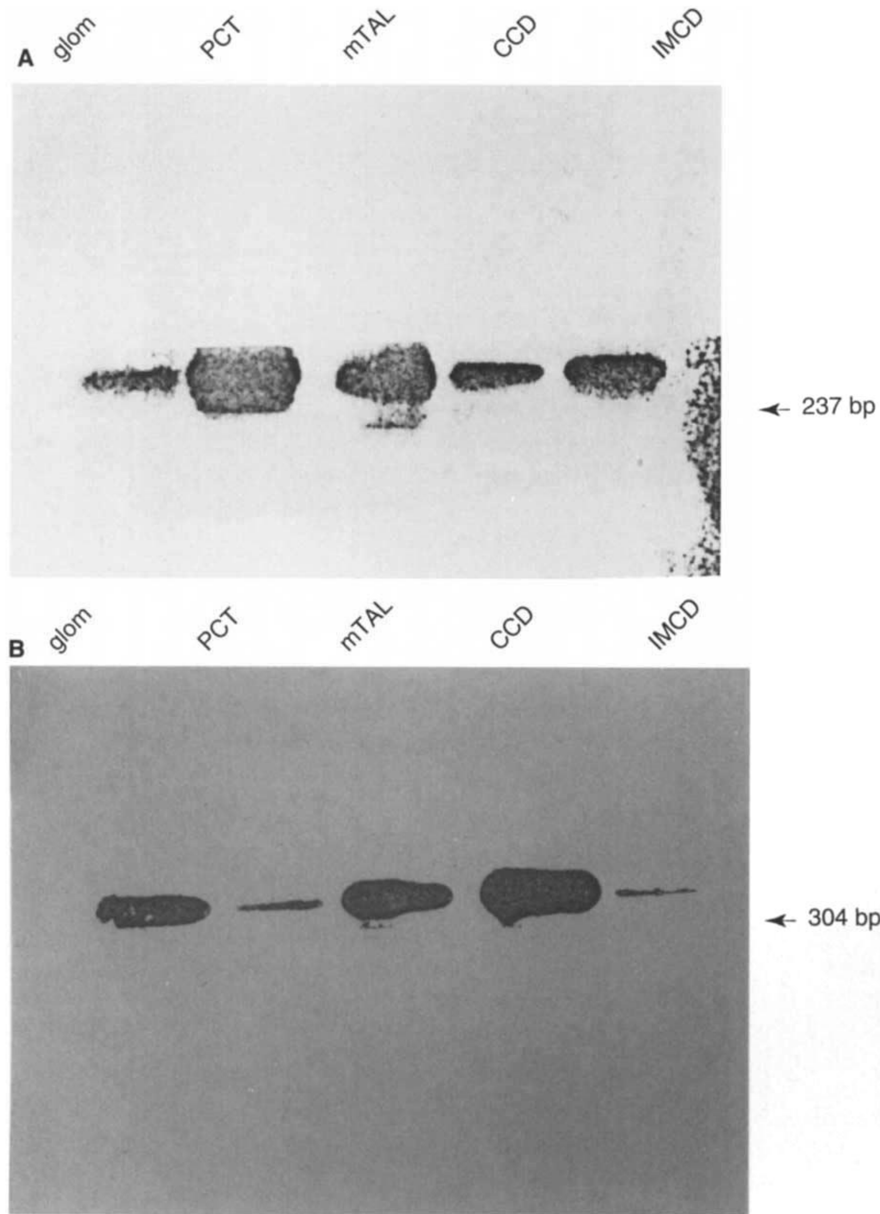

Fig. 6. *Localization of mRNAs encoding Na^+-K^+-ATPase β subunit isoforms in microdissected renal structures.* Southern analysis of size-fractionated of PCR products amplified from the indicated microdissected segments with the β1 (**A**) or β2 (**B**) isoform-specific primers. Molecular weight standards were the 1 kb ladder from GIBCO-BRL. The blots were probed with digoxigenin-labeled cDNAs specific for each β isoform.

abundance of β1 and β2 mRNAs in the cortex, outer and inner medulla. In each parenchymal zone, the β1 and β2 probes protected bands of 271 bp and 304 bp, respectively (Fig. 3). No protection was seen when either probe was hybridized with yeast RNA. Although there was variability in the measurements of radioactivity in the protected bands, β1 subunit mRNA was roughly 20- to 30-fold more abundant than β2 mRNA in the cortex and outer medulla, and 10- to 15-fold more abundant in the inner medulla in each experiment (Fig. 3). The relative levels of β1 transcripts were not significantly different among the three parenchymal zones, but the relative abundance of β2 mRNA in the inner medulla was significantly greater than the levels expressed in renal cortex.

To determine the zonal distribution of α1-T mRNA in the kidney, we performed PCR with the α1-T primers on first strand cDNA prepared from cortex, outer medulla, and inner medulla. As seen in Figure 4, a 389 bp fragment of predicted size was amplified from each of the three zones. To ascertain whether the 389 bp PCR product was identical in molecular structure to the α1-T mRNA characterized in aortic smooth muscle cells [13], the 389 bp fragment was subcloned and sequenced. The nucleotide sequence of the 350 bp internal to the primers of the cloned α1-T PCR product was identical to that of the aortic smooth muscle clone. We did not sequence the minor bands observed on the agarose gel, nor did we quantify α1-T transcript abundance.

Studies on microdissected glomeruli and tubules

To analyze the distribution of Na^+-K^+-ATPase isoform mRNAs along the nephron, we performed RT-PCR with isoform-specific primer pairs on microdissected glomeruli, PCTs, mTALs, CCDs, and IMCDs. In control experiments, direct amplification (without reverse transcription) of the RNAs from each renal

Table 2. Expression of Na^+-K^+-ATPase subunit isoform mRNAs along the nephron

Structure	Na^+-K^+-ATPase subunits					
	α1	α2	α3	α1-T	β1	β2
Glomerulus	5/5	2/5	5/5	3/3	5/5	5/5
PCT	8/8	3/7	8/8	0/4	8/8	6/8
mTAL	5/7	3/10	7/7	0/4	6/7	4/8
CCD	5/5	2/6	5/5	0/4	5/5	3/5
IMCD	4/4	2/9	4/4	0/4	4/4	3/4

Reverse transcription-PCR products from microdissected renal structures were evaluated by gel electrophoresis and (with the exception of α1-T) Southern blots (see Figures 5 and 6, and **Methods**). The values indicate the number of positive results/total number of experiments, in which each experiment was performed on different glomerular or tubular samples obtained from at least three different animals. Samples were recorded as positive if a signal consistent with a PCR product of predicted size was observed by ethidium bromide staining of the size-fractionated products (α1-T) and blot hybridization with the probes specific for the full-length isoforms. Abbreviations are: PCT, proximal convoluted tubule; mTAL, medullary thick ascending limb of Henle; CCD, cortical collecting duct; IMCD, inner medullary collecting duct.

structure with each set of primers produced no reaction product, indicating that the samples did not produce detectable amplification product from genomic DNA. Representative Southern blots and agarose gels are presented in Figures 5 and 6, and the results of all experiments are summarized in Table 2. Amplification with the α1 primers (Fig. 5A) yielded the expected 331 bp band from all segments, although the hybridization signal observed for the glomerular PCR products was relatively weak. As seen in Figure 5B, the α2 primer pairs amplified a single 530 bp band of the expected size from cDNAs synthesized from glomeruli, PCT, CCD, and IMCD. With more prolonged exposure of the blots to the color detection reagents, a faint α2 signal was also observed for the mTAL PCR products. The 313 bp cDNA copy of α3 mRNA was identified in all of the nephron segments, although, as with α2, the mTAL gave the weakest signal (Fig. 5C). α1-T transcripts were identified in microdissected glomeruli, but not in any of the isolated tubule segments (Fig. 5D). PCR with the β1 isoform primers yielded from all five segments a major product migrating at the expected size of 237 bp (Fig. 6A). Southern hybridization of the PCR products amplified with the β2 subunit primers identified a 313 bp (the predicted size) in the cDNA samples from each renal structure (Fig. 6B).

As seen in Table 2, amplified products of diagnostic size were consistently observed for the α1, α3, and β1 isoforms in each of the renal structures examined. β2 mRNA was consistently amplified from all the structures except for the mTAL, for which only half of the experiments yielded a positive result. The most inconsistent results for each nephron segment were obtained with the α2 isoform-specific primers.

Discussion

The RT-PCR technique provides a more sensitive, specific, and potentially quantitative analysis of mRNA expression than do other traditional methods for RNA analysis. When combined with microdissection, it offers the ability to establish not only the molecular identity of the amplified gene product, but also its segmental localization in the nephron. This feature is of particular importance in studying gene expression among several homologous isoforms, such as those of the subunits comprising the Na^+-K^+-ATPase. Moreover, the extreme sensitivity of PCR is required for analysis of mRNAs in such small samples of tissue. Using this approach, combined with dot-blot and RNase protection analyses of steady-state mRNA levels, we demonstrate the coexpression of mRNAs encoding the Na^+-K^+-ATPase α1, α2, α3, β1, and β2 subunits isoforms in isolated glomeruli, PCTs, mTALs, CCDs, and IMCDs, and provide quantitative estimates of transcript abundance for each subunit isoform in the cortex, outer and inner medulla. Moreover, we show that mRNA for the alternative splice variant, α1-T isoform is expressed in each parenchymal zone and isolated glomeruli, and that its nucleotide sequence is identical to the clone identified in aortic smooth muscle cells. These results confirm, by independent methods, and extend our recent *in situ* hybridization studies of Na^+-K^+-ATPase isoform gene expression in the kidney [22], and demonstrate that all of the full-length α and β subunit isoforms are differentially expressed in the zones of the renal parenchyma, and specifically expressed in tubule segments involved in transepithelial transport of sodium and potassium.

Although previous studies consistently observed the presence of α1 and β1 subunit mRNAs in the kidney, the data regarding the expression of the other Na^+-K^+-ATPase subunit isoforms in this organ has been controversial. The bulk of work used Northern analysis of whole kidney RNA to examine isoform gene expression. Some investigators found no expression of α2 [6, 9, 10] or α3 [9, 11] mRNAs, whereas others detected variable levels of α2 [5, 11], α3 [5, 6, 10] or β2 [7] transcripts in the kidney. Several studies reported significant expression of the α2 or α3 transcripts in the kidney. Emanuel et al [5] found more α2 mRNA in kidney than in heart, a tissue generally thought to express significant amounts of this isoform. Herrera et al [6] reported roughly equivalent amounts of a 4.5 kb α3 transcript and α1 mRNA in the kidney. Finally, Sverdlov et al [10] detected greater amounts of α3 mRNA in kidney than in brain, another tissue thought to express this isoform in significant quantities. None of these studies, however, distinguished the zonal distribution of the various isoforms in the kidney.

The data presented here support the view that the α1 and β1 subunits are expressed at much higher levels than the α2, α3, and β2 subunit isoforms in the kidney. In this study, the α1, α2 and α3 isoforms contributed roughly 70%, 20 to 25% and 10%, respectively, of the overall α isoform transcripts in each renal zone, whereas the β1 and β2 subunits contributed ~95% and ~5%, respectively, of the overall β subunit mRNA expression in the kidney. Since by *in situ* hybridization [22] we did not detect significant expression of the α2, α3, or β2 isoform mRNAs in neural or vascular structures within the kidney, we assume that the renal tubules and glomeruli accounted for the bulk of mRNA expression of these isoforms in each zone. Our estimates of α isoform mRNA levels are slightly higher than Hansen's [32] measurements of α isoform protein abundance in microsomes prepared from whole rat kidney. Whereas both studies estimated the levels of the α2 isoform to be about twice that of α3, Hansen reported that the α2 and α3 subunits comprised ~11% and ~5%, respectively, of total α subunit protein. Whether these apparent differences between mRNA and protein abundance reflect methodological differences, less efficient translation of the α2 and α3 isoform mRNAs compared to α1 mRNA, reduced membrane delivery of the α2 and α3 subunit polypeptides, or shorter

half-lives of the α2 and α3 subunit polypeptides remains to be studied.

Our ability to detect, consistently, α2, α3, and β2 subunit mRNAs likely reflects our use of isoform-specific cRNA probes of high specific activity compared to the cDNA probes used by others in earlier studies [5–11]. Given the lack of significant homology among our α and β subunit isoform riboprobes, and the high stringency hybridization and washing conditions used in our solution and blot hybridizations, it is extremely unlikely that cross-hybridization of one isoform with its isotype occurred. Although ideally, we would have used probes of comparable length in the dot-blot analysis, in selecting a shorter α2 probe, we elected to sacrifice some quantitative accuracy to guarantee isoform-specificity. Since the data were corrected for these size discrepancies, and since our principal objective was to obtain only an estimate of the relative abundance of the α isoform transcripts, these results are nonetheless informative. However, absolute quantitation of the transcript levels should not be inferred from these results.

Although our estimates of the regional abundance of the various α and β isoforms do not provide direct information about the levels of Na^+-K^+-ATPase gene expression in the individual tubule segments, comparison of these data with the pattern of renal cellular expression identified in our *in situ* hybridization study [22] suggests some tentative conclusions. In this earlier report, the cellular expression patterns for the α isoforms throughout the kidney were generally similar. Therefore it is not surprising that the various α isoforms, though expressed at different levels compared to one another, were comparably expressed among the major zones of the renal parenchyma. The higher levels of α2 in the inner medulla compared to the cortex must reflect enhanced expression in the IMCD, papillary interstitial cells, or papillary surface epithelium, or reduced expression in the glomerulus, CCD, cTAL, CCD, or connecting segment, since these were the only cell types shown to express α2 mRNA by *in situ* hybridization. Similarly, β2 subunit hybridization signal was particularly prominent in the papillary interstitial cells and papillary surface epithelium when compared to the signal generated by *in situ* hybridization with the β1 riboprobe. Thus, the proportionately higher levels of β2 mRNA in the inner medulla compared to its expression in the other zones likely represent the transcripts contributed by these cells. The fact that we observed a fairly uniform, rather than a segment-specific distribution of the various isoforms in the nephron segments examined in this and our earlier [22] report, suggests that variations in the regulation of renal Na^+-K^+-ATPase may be related not only to variation in subunit isoform abundance, but also to differences in other determinants such as membrane microdomain, distribution of hormonal receptors, or paracrine factors along the nephron. Clearly quantitation of transcript abundance in the individual nephron segments by quantitative PCR methods will further clarify the importance of the different isoforms in specific nephron segments.

Our finding of α1-T transcripts in the three major zones of the renal parenchyma and isolated glomeruli extends the work of Medford et al [13], who detected this isoform in rat aortic smooth muscle cells. These results imply that a common mechanism of α1 RNA processing functions in these tissues. Thus RNA processing appears to be another mechanism governing and coordinating Na^+-K^+-ATPase biosynthesis in the kidney, and another mechanism producing α isoform heterogeneity in this organ. The precise biological role of this isoform has not been established, but it is presumed to function as an ATPase, at least in vascular smooth muscle cells [13]. Given its expression in vascular smooth muscle cells and, in preliminary data, cultured glomerular mesangial cells [33], but not in tubular epithelial cells, it is intriguing to speculate that the α1-T isoform subserves a role unique to contractile cell function. The absence of detectable transcripts for this isoform in the isolated tubules indicates that α1-T does not contribute to overall Na^+-K^+-ATPase function in these segments. Although the detection of α1-T mRNA in the outer and inner medulla may simply reflect expression of this isoform in vasculature coursing through these zones, it is possible that α1-T is expressed in other nephron segments (such as, S3 segment of the proximal tubule, outer medullary collecting duct, thin descending limbs of Henle) not examined in our study.

Our results suggest the potential for the intrarenal expression of up to eight structurally unique α-β subunit isoform complexes (assuming that the α1-T subunit forms a heterodimer), and, thus, of multiple Na^+-K^+-ATPase isoenzymes. Our previous work established that the α1, α2, α3 subunits are capable of stable assembly with the β1 [34] and β2 (M. Lemas, K. Takeyasu, B. Kone, and D.M. Fambrough, manuscript submitted for publication) subunits of the Na^+-K^+-ATPase to yield heterodimers. The compelling physiological and biochemical evidence for functional heterogeneity of the Na^+-K^+-ATPase along the nephron [18–21] argues for the renal expression of multiple isoenzymes expressed at physiologically significant levels. For example, the ouabain binding affinity of the Na^+-K^+-ATPase varies significantly along the nephron, with higher affinity in the CCD than in more proximal segments [19, 20]. Moreover, the K_m for Na^+ of the Na^+-K^+-ATPase expressed in the CCD is threefold higher than that of the PCT or cTAL [18]. The coexpression of α1, α2, α3, β1, and β2 subunit mRNAs in the CCD, reported here, provides the molecular basis with which to explain these findings, as well as the inhibitory effect of an α3-specific antibody on CCD Na^+-K^+-ATPase activity [21].

The potential physiological importance of multi-gene expression of the Na^+-K^+-ATPase subunits in the kidney is significant. Endogenous ouabain has been postulated to modulate Na^+-K^+-ATPase activity *in vivo* and to play a role in the regulation of cell Na^+ balance and whole body salt and water homeostasis [reviewed in 34]. Biochemical studies have shown that the principal functional property that differentiates the α isoforms of the rat Na^+-K^+-ATPase is their affinity for ouabain [15]. Since the ouabain binding affinity of the α2 and α3 isozymes is about three orders of magnitude higher than that of Na^+-K^+-ATPase comprised of the α1 subunit [15], variations in the expression of different isozymes along the rat nephron may allow for fine control of renal Na^+-K^+-ATPase activity by endogenous ouabains under physiologic or pathophysiologic conditions. Moreover, recent functional studies suggest that different combinations of a given α subunit with one or the other β subunit (for example, α1/β1 vs. α1/β2) may give rise to enzymes with different kinetic properties. For example, biochemical studies of rat pineal gland Na^+-K^+-ATPase, which appears to be comprised of α3/β2 subunits, has a higher affinity for Na^+ than does the α1-containing isoenzyme [17]. In contrast, transfection of β1-containing HeLa cells with the rat α3 cDNA resulted in the expression of Na^+-K^+-ATPase molecules with a two- to threefold lower apparent affinity for Na^+ compared to cells transfected with the rat α1 isoform

[16]. Thus, the $\alpha3/\beta2$ isoenzyme may operate more efficiently when intracellular Na^+ concentrations are reduced, whereas the $\alpha3/\beta1$ may be activated only when intracellular Na^+ concentrations are high. Since intracellular Na^+ is a key short-term regulator of Na^+-K^+-ATPase activity, and thus of Na^+ reabsorption by the renal tubules, the ability to express selectively Na^+-K^+-ATPase with unique kinetic sensitivities to intracellular Na^+ levels would provide the tubules with an array of mechanisms with which to respond to changing ion transport demands, as might occur during variations in salt and fluid balance. Similarly, the $\alpha2$ isoform, which based on our estimates is likely to contribute more significantly than the $\alpha3$ isoform to renal Na^+-K^+-ATPase expression, has been postulated to operate efficiently when extracellular K^+ concentrations are high [36]. Finally, given the apparent functional differences among the various α isoforms [14–17] and differences in their 5′ regulatory elements [15], induction by a hormone, *trans*-acting factor, other effector molecule, or pathophysiologic state (such as nephrotic syndrome, [19]) of a specific isoform in a given nephron segment could have important physiological implications.

Given the extreme sensitivity of PCR and the limitations of renal microdissection, we cannot exclude the possibility that "contamination" by other cell types (such as interstitial cells) adherent to the microdissected segments contributed to the amplified products. However the congruence of the present results with our *in situ* hybridization data [22], in which expression of the various isoforms in the glomeruli, PCT, mTAL, CCD, and IMCD was demonstrated, strongly suggests that the RT-PCR products were derived principally from subunit isoform genes expressed in the specific microdissected structures and not from adherent cells. Since we detected transcripts encoding the α and β subunit isoforms in each zone by Northern hybridization and RNase protection assays (methods far less sensitive than PCR), it is also unlikely that the RT-PCR products represented simply illegitimate transcripts.

Given the limitations of quantitation by PCR, and the minute amounts of RNA isolated from the tubules, we did not attempt absolute quantitation of the PCR products. It is perhaps noteworthy, however, that PCR with the $\alpha1$, $\alpha3$, and $\beta1$ primers, using equivalent aliquots of cDNA from a given segment as template, routinely yielded the greatest signals on ethidium-bromide stained gels and Southern blots. However, since we did not control for amplification efficiencies in the PCRs, we cannot determine from these data whether the differences in amounts of PCR products necessarily reflect variations in mRNA abundance among the different Na^+-K^+-ATPase isoforms. The fact that the $\alpha2$ isoform was detected less consistently than the other isoforms in each microdissected structure by RT-PCR, yet was more abundant in the parenchymal zones than the $\alpha3$ and $\beta2$ isoforms as assessed by blot and solution hybridizations, might indicate increased susceptibility of $\alpha2$ mRNA (relative to the other isoforms) to degradation (the chances of degradation are much greater in the more prolonged microdissection procedure) or less efficient PCR with the $\alpha2$ primer pairs than with those for the other isoforms.

We found the PCR method for labeling the cloned PCR products with digoxigenin [31] to be simple, accurate, reproducible, and inexpensive (since it obviated the need for synthesis of additional internal oligonucleotides to use as hybridization probes). Even though the primer sequences were included as part of the probe, they contributed only 10 to 25% of the probe sequence; thus it is extremely unlikely that the probes cross-hybridized under such high stringency conditions to another molecular species of the same predicted size.

In summary, the present study demonstrates that the RT-PCR technique can be used to detect the expression of genes encoding the various α and β subunit isoforms of the Na^+-K^+-ATPase in microdissected nephron segments of the rat. To our knowledge, this investigation represents the first report of the identification of all the known α and β subunit isoform mRNAs in each of the microdissected renal structures examined,[1] and the first quantitative analysis of α and β isoform mRNA abundance in the zones of the renal parenchyma. The expression of multiple Na^+-K^+-ATPase subunit isoforms and of potential α-β isoform complexes in the kidney endows its various cell types with significant versatility in managing cation balance at the level of both the cell and the organism. The data presented here also point to the potential for multiple control points for regulation of specific Na^+-K^+-ATPase isoenzymes along the nephron, including rates of isoform gene transcription, RNA processing (giving rise to $\alpha1$-T transcripts), and efficiency of α-β subunit assembly. Our current efforts are directed towards understanding these regulatory pathways.

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