J Am Soc Nephrol 10: 230–237, 1999

# Cloning and Characterization of Two New Isoforms of the Rat Kidney Urea Transporter: UT-A3 and UT-A4

ALEXANDER KARAKASHIAN,* RICHARD T. TIMMER,† JANET D. KLEIN,‡ ROBERT B. GUNN,† JEFF M. SANDS,‡ and SERENA M. BAGNASCO*

**Department of Pathology, †Department of Physiology, and ‡Renal Division, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia.*

*Abstract.* Urea transport in the kidney is important for the production of concentrated urine and is mediated by a family of transporter proteins, identified from erythropoietic tissue (UT-B) and from kidney (UT-A). Two isoforms of the renal urea transporter (UT-A) have been cloned so far: UT-A1 and UT-A2. We used rapid amplification of cDNA ends to clone two new isoforms of the rat UT-A transporter: UT-A3 and UT-A4. UT-A3 and UT-A4 are 87% homologous. The UT-A3 cDNA encodes a peptide of 460 amino acids, which corresponds to the amino-terminal half of the UT-A1 peptide and is 62% identical to UT-A2. The UT-A4 cDNA encodes a peptide of 466 amino acids, which is 84% identical to UT-A2. Transient transfection of HEK-293 cells with the UT-A3 or UT-A4 cDNA results in phloretin-inhibitable urea uptake, which is increased by forskolin. Thus, both new isoforms encode functional urea transporters that may be vasopressin-regulated. UT-A3 and UT-A4 mRNA are expressed in the renal outer and inner medulla but not in the cortex; unidentified UT-A isoforms similar to UT-A3 may also be expressed in the testis. It is concluded that there are at least four different rat UT-A urea transporters.

Urea is a major end product of protein metabolism. Although urea can cross cell membranes slowly by passive diffusion, its transport rate in kidney and erythrocytes is enhanced by specific transporter proteins (1). In the kidney, urea transport is important for the production of concentrated urine. The first evidence for a vasopressin-regulated, facilitated urea transport process in a mammalian inner medullary collecting duct (IMCD) segment was reported about 10 yr ago (2). The function of this renal vasopressin-regulated urea transporter has been further characterized in subsequent studies (3–9). The cDNA sequence of the first renal urea transporter UT2 was identified by expression cloning from rabbit inner medulla (10). The rat and human cDNA for this form of the urea transporter have also been cloned (11,12). A longer cDNA isoform, UT1, was later cloned, which appears to be a vasopressin-regulated urea transporter in the rat kidney IMCD (13). The cDNA of the human erythrocyte urea transporter (HUT11) was initially cloned from human bone marrow cells (14), and homologous cDNA for the rat erythrocyte urea transporter (rUT11, UT3) have been cloned subsequently (15,16), and are found to be expressed in kidney, brain, and testis.

Received July 13, 1998. Accepted August 30, 1998.

This work was presented in part at the 30th annual meeting of the American Society of Nephrology, November 2 to 5, 1997, San Antonio, Texas, and published in abstract form (*J Am Soc Nephrol* 8: 15A, 1997).

Correspondence to Dr. Serena M. Bagnasco, Department of Pathology, Emory University School of Medicine, WMB Room 7105 C, 1639 Pierce Drive, Atlanta, GA 30322. Phone: 404-727-4026; Fax: 404-727-8540; E-mail: sbagnas@emory.edu

1046-6673/1002-0230$03.00/0

Journal of the American Society of Nephrology

Copyright © 1999 by the American Society of Nephrology

The physiologic and molecular characterization of these urea transporters shows that they are remarkably homologous, and therefore they may be regarded as a family, similar to the aquaporins. A new, systematic nomenclature has recently been proposed for the urea transporters (1) and will be used in this report. The urea transporter family includes two main groups: the renal urea transporters (UT-A) and erythrocyte urea transporters (UT-B), which are likely to be encoded by different genes (reviewed in reference (1)). There are probably other urea transporters, which have not been identified. For example, the two known isoforms of the renal urea transporter UT-A1 (UT1) and UT-A2 (UT2), which are expressed in the renal tubule (17,18), are localized only to the apical membrane of IMCD cells and thin descending limb (18,19). Thus, they cannot account for the phloretin-inhibitable urea transport described in the basolateral membrane of IMCD (20), which must occur through a different, as yet unidentified urea carrier.

Using cloning by rapid amplification of cDNA ends (RACE), we searched for new members of the urea transporter family. We report two new cDNA isoforms of the renal UT-A urea transporter: UT-A3 and UT-A4, cloned from the rat inner medulla, and the partial characterization of their functional properties in a heterologous expression system.

## Materials and Methods

### *Rapid Amplification of cDNA Ends*

3′ RACE was performed using the reagents provided in the 5′/3′ RACE kit (Boehringer-Mannheim, Indianapolis, IN), following the protocols suggested by the manufacturer. Briefly, first-strand cDNA was synthesized from 2.5 mg of total RNA from rat renal

medulla (TRI Pure Isolation Reagent, Boehringer-Mannheim), using an oligo(dT)-anchor primer (5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT-3′) and avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated for 60 min at 55°C, followed by incubation at 65°C for 10 min. Amplification by PCR was performed on 1.5 ml of the cDNA, using Expand™ High Fidelity PCR System (Boehringer-Mannheim), the PCR anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′), and the sense primer PA (5′-GCATCGCATTGACATCTTCCTAC-3′), corresponding to nucleotides 210 to 232 of the UT-A1 cDNA (13), for 10 cycles of 1 min at 94°C, 1 min at 58°C, 3 min at 72°C, followed by 25 cycles of 45 s at 94°C, 1 min at 58°C, 3 min + 20 s/cycle at 72°C. A second PCR amplification was performed using the sense primer PB (5′-CTTCACATGACAGATGAGCGAC-3′), corresponding to nucleotides 364 to 385 of UT-A1 cDNA, and the PCR anchor primer. The products of the second PCR amplification were gel-purified and sequenced using AmpliTaq DNA Polymerase FS (ABI PRISM Dye Terminator Cycle sequencing kit, Perkin Elmer, Foster City, CA). Sequences were analyzed on an automatic DNA sequencing system (ABI PRISM Genetic Analyzer, Perkin Elmer). 5′ RACE was performed using a similar protocol with nested antisense primers corresponding to nucleotides 600 to 631, and 481 to 457 of the UT-A1 cDNA. The resulting PCR products were gel-purified and sequenced to verify that the 5′ end sequences of the UT-A3, UT-A4, and UT-A1 cDNA were identical.

To exclude that discrepancies in the sequence of otherwise identical cDNA segments among the new isoforms and the previously published sequence of UT-A1 (13) could result from artifact, we amplified the UT-A1 cDNA by reverse transcription (RT)-PCR from total RNA from rat inner medulla, and compared its sequence with the sequences of UT-A3 and UT-A4 cDNA as well as with the sequence of the corresponding segments of PCR-amplified rat genomic DNA.

### *In Vitro Transcription-Translation and Immunoblotting*

The coding region of the cDNA of UT-A1 (nucleotides 493/3262), UT-A2 (nucleotides 816/2283), UT-A3 (nucleotides 493/1790), and UT-A4 (nucleotides 493/2217) was subcloned into the *Hin*dIII, *Xho*I-digested mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), under the control of the T7 promoter. *In vitro* translation from the above constructs was performed using a coupled transcription/translation reagent system (TNT Coupled Reticulocyte Lysate System, Promega, Madison, WI) in the presence of L-[$^{35}$S]-methionine (1000 Ci/mmol, Amersham, Arlington Heights, IL), following the protocol suggested by the manufacturer, in the absence and in the presence of canine pancreatic microsomes (Promega). The products obtained were analyzed by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, followed by autoradiography. For Western blotting, the translated products were size-separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Gelman Scientific, Ann Arbor, MI). Blots were incubated with a primary rabbit polyclonal antibody generated toward the carboxy-terminal portion of rat UT-A1 and UT-A2 (21), followed by a secondary goat anti-rabbit IgG, and the bound antibody was visualized using chemiluminescence (ECL kit, Amersham).

### *cDNA Expression and Cell Transfection*

To test the function of UT-A3 and UT-A4 as urea transporters, the coding regions of UT-A3 cDNA (364/1790) and UT-A4 cDNA (364/2217) were subcloned in pcDNA3. Similar pcDNA3 constructs for UT-A2 cDNA (816/2283) were used for functional comparison. These constructs were transiently transfected into the human embryonic kidney cell line HEK-293. HEK-293 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 31, and used until passage 45, after which fresh cultures were started from frozen passage 32 cells. Cells were maintained in T75 flasks, in 5% $CO_2$/95% air in a 37°C incubator, with minimum essential medium, L-glutamine, and 5% fetal calf serum. Cells (2 to 3 $\times$ $10^5$/cm$^2$) were transfected with the calcium phosphate transfection system kit (Life Technologies, Gaithersburg, MD), using 30 to 40 $\mu$g of plasmid construct DNA per flask. After 4 h, cells were subjected to glycerol shock for 2 min with 2 ml of 18% glycerol at room temperature, followed by replacement with fresh medium. The next day cells were plated in 24-well plates (Corning, Marietta, GA) at a density of 1.6 $\times$ $10^5$ cells/well. Flux measurements were carried out at 48 $\pm$ 6 h after transfection.

### *Northern Hybridization Analysis and RT-PCR Analysis of mRNA Expression*

A rat multiple tissue Northern blot (Clontech, Palo Alto, CA) was hybridized with $^{32}$P-labeled DNA probes (Prime-A-Gene kit, Promega). The following cDNA segments were amplified by PCR, labeled, and used as DNA probes in sequential hybridizations of the same membrane to identify the different isoforms: probe P1, corresponding to nucleotides 26 to 481 of UT-A1 cDNA, is expected to hybridize to transcripts that share this same sequence, *i.e.*, UT-A1, UT-A3, and UT-A4; probe P2 includes nucleotides 63 to 492 of UT-A2 cDNA, specific for the UT-A2 transcript only; probe P3 includes nucleotides 1764 to 1986 of UT-A3 cDNA, specific for UT-A3 only. Hybridization (65°C), washings (0.1$\times$ SSC, 0.1% SDS, 50°C), and stripping of the probes from the membrane for multiple hybridizations were performed according to the protocols suggested by the manufacturer.

For RT-PCR analysis of mRNA expression, rat kidney tissue was dissected into cortex, outer medulla, and inner medulla and homogenized for extraction of total RNA using TRI Pure Isolation Reagent (Boehringer-Mannheim). Reverse transcription from 1 $\mu$g of total RNA and amplification of the first-strand cDNA were performed using the Super Script One-Step RT-PCR System (Life Technologies), following the protocol suggested by the manufacturer. DNA sequence analysis was performed using the Wisconsin Sequence Analysis Software Package, version 9.1-UNIX (Genetic Computer Group, Madison, WI).

### *Measurement of [$^{14}$C]-Urea Transport*

Cells were washed with 0.5 ml of $HCO_3$-free, Hepes-buffered minimum essential medium containing (in mM): 5 urea, 143 NaCl, 4.36 KCl, 1.8 $CaCl_2$, 0.81 $MgCl_2$, 5.55 D-glucose, 25 Hepes, pH 7.64, at 20°C, preincubated at 37°C in room air for 30 min, and transferred on a water thermostated platform at 21 $\pm$ 2°C. At known intervals, the preincubation medium was substituted with medium containing 2 $\mu$Ci/ml $^{14}$C-urea (Amersham) with or without phloretin (50 to 250 mM; Sigma, St. Louis, MO). The influx was stopped simultaneously for all wells by three rapid washes with ice-cold solution containing (in mM): 150 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, and 0.05 phloretin. The cells in each well were solubilized in 0.5 ml of 25 mM NaOH with 0.5% deoxycholate; 100 ml from each well was used to measure protein with BCA assay (Pierce, Rockford, IL), and 500 $\mu$l was counted in 3 ml of Optifluor (Packard Instruments, Meriden, CT) in a liquid scintillation counter.

### *Statistical Analyses*

The net cpm/mg protein *versus* the elapsed exposure time to the tracer (15, 5, 2.5, 1.5, and 0.5 min) were fit to a straight line by the least squares method or a single exponential. The initial slope of the fit line divided by the specific activity (cpm/nmol) was the calculated flux: nmol/g protein per minute. The standard error of each flux value was calculated by dividing the standard error of the slope by the specific activity. The influx was linear for 5 min for urea influx measurements. Samples were measured in cells transfected with vector containing the cDNA as well as in the cells sham-transfected with vector only. Measurements of urea influx were repeated four times in separate experiments, using different batches of transfected cells. Differences were considered statistically significant for $P < 0.05$, using unpaired two-tailed *t* test.

## Results

### *Cloning of UT-A3 and UT-A4 cDNA*

Our approach in searching for new isoforms of the rat urea transporter UT-A was based on cloning by rapid amplification of cDNA ends (RACE) (22). The amplification products of the first-strand cDNA generated by 3′ RACE revealed a 3.8-kb band corresponding to UT-A1, plus two smaller products of approximately 1.9 and 2.4 kb, which were designated as UT-A3 and UT-A4, respectively, and sequenced. 5′ RACE was performed to obtain the 5′ end of the new cDNA and to confirm that the sequences of UT-A3, UT-A4, and UT-A1 cDNA (which we reverse-transcribed from rat inner medulla) had identical 5′ ends.

UT-A3 cDNA has an open reading frame of 1380 nucleotides (Figure 1A), equivalent to a peptide of 460 amino acids with a predicted molecular weight of 50 kD. UT-A3 cDNA is identical to the nucleotide sequence corresponding to the N-terminal half of UT-A1, but diverges from UT-A1 cDNA in the codon for UT-A3's last amino acid ($D_{460}$ replaces $G_{460}$). UT-A3 also has a unique 3′ untranslated region (3′ UTR), which is not common to any other UT-A isoform. To validate this part of the cDNA sequence, it was compared and found to be identical to the corresponding sequence of rat genomic DNA.

UT-A4 cDNA has an open reading frame of 1398 nucleotides (Figure 1B), encoding a peptide of 466 amino acids, with a predicted molecular weight of 51 kD, which is 87% homologous to the UT-A3 peptide sequence. The cDNA of UT-A4 is identical to UT-A1 in the first 1100 nucleotides; the remaining 1485 nucleotides correspond to the 3′ terminal cDNA of UT-A1, suggesting that it results from alternative splicing between 5′ terminal and 3′ terminal groups of exons in the UT-A gene. A schematic representation of the cDNA of the four UT-A isoforms is shown in Figure 2A. The nucleotide sequence of the regions that UT-A3, UT-A4, and UT-A1 have in common are identical among the cDNA that we generated. However, compared with the UT-A1 cDNA sequence originally reported (13), we find consistent nucleotide discrepancies, resulting in the following amino acid differences: $L_{17}$ replaces $P_{17}$ and $G_{96}$ replaces $A_{96}$. Since in our hands the cDNA and genomic DNA sequences of these codons are also identical, the differences may be due to polymorphisms.

Both UT-A3 and UT-A4 peptides have a consensus glycosylation site at $Asn_{279}$, and UT-A4 has an additional consensus site at $Asn_{357}$. UT-A3 has two consensus sequences for cAMP-dependent phosphorylation sites at $Ser_{81,88,}$ and four for protein kinase C (PKC) phosphorylation sites at $Ser_{23,79,205}$ and $Thr_{447}$. UT-A4 has three consensus sequences for cAMP-dependent phosphorylation sites at $Ser_{81,88,452}$, and three for PKC phosphorylation sites at $Ser_{23,79,205}$.

We have analyzed the sequences of these new urea transporter isoforms, as well as the other known urea transporters, and, based on previously stated criteria (1), we propose the structural models for UT-A3 and UT-A4 which are shown in Figure 2B; those for UT-A1 and UT-A2 are shown for comparison. The models propose a symmetrical configuration in which an intracellular amino-terminal domain is followed by three transmembrane segments, a large extracellular loop containing an N-glycosylation site, three more segments that completely span the membrane, a smaller integral membrane domain, and an intracellular carboxy-terminal domain. Consensus phosphorylation sites for protein kinase A and PKC, potentially important in the regulation of activity, are within the intracellular compartment.

### *In Vitro Translation*

The *in vitro* translation products of the UT-A3 and UT-A4 cDNA subcloned into pcDNA3 are shown along with those of UT-A1 and UT-A2 in Figure 3A. The UT-A3 construct yielded a product with an apparent molecular weight of approximately 35 kD, whereas the UT-A4 construct yielded a product of approximately 37 kD. An increase in size of approximately 4 kD was observed when UT-A3 and UT-A4 were translated in the presence of pancreatic microsomes, presumably reflecting *in vitro* glycosylation of the two proteins (Figure 3B). As expected, only UT-A4 was recognized when both UT-A3 and UT-A4 translation products were tested by immunoblotting with an antibody directed toward the last 20 amino acids of UT-A1/UT-A2 carboxy terminus (21,18) (Figure 3C).

### *Functional Analysis of UT-A3 and UT-A4 Proteins*

The functional activity of UT-A3 and UT-A4 proteins as urea transporters was analyzed by measuring the influx of $^{14}$C-urea in HEK-293 cells transiently transfected with pcDNA3-UT-A3 or pcDNA3-UT-A4, and their activity compared with those of HEK-293 cells transfected with pcDNA3 only, as negative control, and with pcDNA3-UT-A2, as a positive control. HEK-293 cells were chosen because they do not express UT-A transporters (23); therefore, any phloretin-inhibitable urea uptake must be from newly synthesized protein after transfection with the above constructs. Cells transfected with either UT-A3 or UT-A4 display phloretin-inhibitable urea transport above control level (sham-transfected cells), and the transport activities of these new isoforms appear comparable to those of UT-A2 (Figure 4A).

Since both UT-A3 and UT-A4 have consensus sites for cAMP-dependent phosphorylation, we tested the effects of inducing intracellular accumulation of cAMP on the rates of urea transport of transiently transfected cells, as an indicator of potential susceptibility to vasopressin regulation.

**A**

```
1    aaaaagaggaaagacagagccggcctagacagcagacatcaggacacccttctaggccag   60
                    M  S  D  H  P  L  K  E  M  S  D  N  N  R  S
61   ctctgttttctgccttaatgctcaaggaagactgcaagatcatctgaggtgggtcctgta  120
     P  L  L  P  E  P  L  S  S  R  Y  K  L  Y  E  S  E  L  S  S
121  acagcgtcagctccacaaagtcatcgctgcaaagttcaggagatgggagatctttcctgt  180
     P  T  W  P  S  S  S  Q  D  T  H  P  A  L  P  L  L  E  M  P
181  tttcttactgagtgtttccgggtgtgagtgcatgccattgacatcttcctacctctgtgg  240
     E  E  K  D  L  R  S  S  D  E  D  S  H  I  V  K  I  E  K  P
241  acaaacaaggacaaggctcgaatcccctcagtctacttcatcagccggacctgcatgact  300
     N  E  R  S  K  R  R  E  S  E  L  P  R  R  A  S  A  G  R  G
301  ggctattttgaccacttgaagtcatctaaatagaagttctcaagaccaccaagaccaagg  360
     G  F  S  L  F  Q  A  V  S  Y  L  T  G  D  M  K  E  C  K  N
361  acacttcacatgacagatgagcgaccatcccctgaaggagatgtctgacaacaaccgcag  420
     W  L  K  D  K  P  L  V  L  Q  F  L  D  W  V  L  R  G  A  A
421  ccccctcttgccagagcctctttccagcagatacaagttgtatgagtcagagctaagcag  480
     Q  V  M  F  V  N  N  P  L  S  G  L  I  I  F  I  G  L  L  I
481  ccctacctggccttcaagctcccaggatacacacccagcccttcccctgctggaaatgcc  540
     Q  N  P  W  W  T  I  A  G  A  L  G  T  V  V  S  T  L  A  A
541  tgaagaaaaggatctccgatcatcggatgaagacagccacatagtgaagattgaaaagcc  600
     L  A  L  S  Q  D  R  S  A  I  A  S  G  L  H  G  Y  N  G  M
601  caacgagcggagtaaacggagagaaagcgagttgccccgcagggcttctgcaggccgggg  660
     L  V  G  L  L  V  A  V  F  S  E  K  L  D  Y  Y  W  W  L  L
661  aggcttcagcctcttccaagctgtgagctacctcactggtgacatgaaggaatgcaagaa  720
     F  P  V  T  F  A  S  M  A  C  P  V  I  S  S  A  L  S  T  V
721  ctggctgaaagataagcccctggtccttcagttcctagactgggtcctgcggggagcagc  780
     F  A  K  W  D  L  P  V  F  T  L  P  F  N  I  A  L  T  L  Y
781  tcaggtgatgtttgtcaacaacccccttagtgggctcatcatcttcatagggctcctgat  840
     L  A  A  T  G  H  Y  N  L  F  F  P  T  T  L  V  K  P  A  S
841  ccagaatccctggtggacgattgctggggcactggggacagtggtctcaaccttggctgc  900
     S  A  P  N  I  T  W  S  E  I  E  M  P  L  L  L  Q  T  I  P
901  ccttgccttgagccaggacaggtcagccatcgcctctggactccatgggtacaacgggat  960
     V  G  V  G  Q  V  Y  G  C  D  N  P  W  T  G  G  V  I  L  V
961  gctggtgggactcctggtggctgtcttctcagagaagttagactactactggtggcttct 1020
     A  L  F  I  S  S  P  L  I  C  L  H  A  A  I  G  S  I  V  G
1021 gtttcctgtgaccttcgcatccatggcctgcccagttatttctagtgccttgagcaccgt 1080
     L  L  A  A  L  T  V  A  T  P  F  E  T  I  Y  T  G  L  W  S
1081 cttcgccaagtgggacctgccagtcttcacactgcccttcaacatcgccttaacactgta 1140
     Y  N  C  V  L  S  C  V  A  I  G  G  M  F  Y  V  L  T  W  Q
1141 cctggcagctacgggccactacaacctttttcttccccacgacgcttgtgaagcctgcgtc 1200
     T  H  L  L  A  L  V  C  A  L  F  C  A  Y  T  G  A  A  L  S
1201 ttcagcgcccaacatcacctggtcagagattgagatgcctttgctgttacaaaccatccc 1260
     N  M  M  A  V  V  G  V  P  P  G  T  W  A  F  C  L  S  T  L
1261 cgttggggtgggccaggtctacggctgtgacaacccctggacaggcggcgtgatcctggt 1320
     T  F  L  L  L  T  S  N  N  P  G  I  H  K  L  P  L  S  K  V
1321 ggctctgtttatctcctctcccctcatctgtttgcacgcagccataggatccattgtggg 1380
     T  Y  P  E  A  N  R  I  Y  F  L  T  A  K  R  S  D  E  Q  K
1381 gctgctggcagcactgacggtggccactcccttcgagacgatctacacgggccttttggag 1440
     P  P  N  G  D
1441 ctacaactgtgtcctctcctgtgtcgccattggaggcatgttctacgtactcacctggca 1500
1501 gacacacctgttggcacttgtctgtgccctgttctgtgcgtacacgggagcggccctctc 1560
1561 caatatgatggcagtggttggtgtgccaccgggcacctgggccttctgtctctccaccct 1620
1621 caccttccttctcctcacaagcaacaaccctggcatccacaagctcccactcagcaaagt 1680
1681 cacctacccagaggccaaccgcatctacttcctgacagcgaaacgcagtgacgagcagaa 1740
1741 gccccccaatggtgactagttcagcgccaagccaaaatgttctgctttgtctgcatccct 1800
1801 atggtctcaactctgggccaagcgacccggccagcactcactttcctcgcctctccttgc 1860
1861 acctgtttcaaatcaaacccacctattcctccctaatcccgaggcgattcgttccctctg 1920
1921 cccagatctttataacaacccctgtagcattaaaggaagctggtacgacaaaaaaaaaa 1980
1981 aaaaa  1985
```

**B**

```
1    aaaaagaggaaagacagagccggcctagacagcagacatcaggacacccttctaggccag   60
                    M  S  D  H  P  L  K  E  M  S  D  N  N  R  S
61   ctctgttttctgccttaatgctcaaggaagactgcaagatcatctgaggtgggtctctgta  120
     P  L  L  P  E  P  L  S  S  R  Y  K  L  Y  E  S  E  L  S  S
121  acagcgtcagctccacaaagtcatcgctgcaaagttcaggagatgggagatctttcctgt  180
     P  T  W  P  S  S  S  Q  D  T  H  P  A  L  P  L  L  E  M  P
181  tttcttactgagtgtttccgggtgtgagtgcatcgcattgacatcttcctacctctgtgg  240
     E  E  K  D  L  R  S  S  D  E  D  S  H  I  V  K  I  E  K  P
241  acaaacaaggacaaggctcgaatcccctcagtctacttcatcagccggacctgcatgact  300
     N  E  R  S  K  R  R  E  S  E  L  P  R  R  A  S  A  G  R  G
301  ggctattttgaccacttgaagtcatctaaatagaagttctcaagaccaccaagaccaagg  360
     G  F  S  L  F  Q  A  V  S  Y  L  T  G  D  M  K  E  C  K  N
361  acacttcacatgacagatgagcgaccatcccctgaaggagatgtctgacaacaaccgcag  420
     W  L  K  D  K  P  L  V  L  Q  F  L  D  W  V  L  R  G  A  A
421  cccccttcttgccagagcctctttccagcagatacaagttgtatgagtcagagctaagcag  480
     Q  V  M  F  V  N  N  P  L  S  G  L  I  I  F  I  G  L  L  I
481  ccctacctggccttcaagctcccaggatacacacccagcccttcccctgctggaaatgcc  540
     Q  N  P  W  W  T  I  A  G  A  L  G  T  V  V  S  T  L  A  A
541  tgaagaaaaggatctccgatcatcggatgaagacagccacatagtgaagattgaaaagcc  600
     L  A  L  S  Q  D  R  S  A  I  A  S  G  L  H  G  Y  N  G  M
601  caacgagcggagtaaacggagagaaagcgagttgccccgcagggcttctgcaggccgggg  660
     L  V  G  L  L  V  A  V  F  S  E  K  L  D  Y  Y  W  W  L  L
661  aggcttcagcctcttccaagctgtgagctacctcactggtgacatgaaggaatgcaagaa  720
     F  P  V  T  F  A  S  M  A  C  P  V  I  S  S  A  L  S  T  V
721  ctggctgaaagataagcccctggtccttcagttcctagactgggtcctgcggggagcagc  780
     F  A  K  W  D  L  P  V  F  T  L  P  F  N  I  A  V  T  L  Y
781  tcaggtgatgtttgtcaacaacccccttagtgggctcatcatcttcataggggctcctgat  840
     L  A  A  T  G  H  Y  N  L  F  F  P  T  K  L  L  Q  P  A  V
841  ccagaatccctggtggacgattgctggggcactggggacagtggtctcaaccttggctgc  900
     Q  N  P  W  W  T  I  A  G  A  L  G  T  V  V  S  T  L  A  A
901  ccttgccttgagccaggacaggtcagccatcgcctctggactccatgggtacaacgggat  960
     L  A  L  S  Q  D  R  S  A  I  A  S  G  L  H  G  Y  N  G  M
961  gctggtgggactcctggtggctgtcttctcagagaagttagactactactggtggcttct 1020
     L  V  G  L  L  V  A  V  F  S  E  K  L  D  Y  Y  W  W  L  L
1021 gtttcctgtgaccttcgcatccatggcctgcccagttatttctagtgccttgagcaccgt 1080
     F  P  V  T  F  A  S  M  A  C  P  V  I  S  S  A  L  S  T  V
1081 cttcgccaagtgggacctgccagtcttcacactgcccttcaacatcgccgtgaccctgta 1140
     F  A  K  W  D  L  P  V  F  T  L  P  F  N  I  A  V  T  L  Y
1141 cctggcagccacgggccactacaatctcttcttccccacgaagctgctgcagcctgcagt 1200
     L  A  A  T  G  H  Y  N  L  F  F  P  T  K  L  L  Q  P  A  V
1201 taccacacccaacatcacctggtcagatgtccaagtgcctttgcttctgagagccatccc 1260
     T  T  P  N  I  T  W  S  D  V  Q  V  P  L  L  L  R  A  I  P
1261 cgttggaatcggccaagtgtatggctgtgacaatccctggactggaggcatcttcctcgt 1320
     V  G  I  G  Q  V  Y  G  C  D  N  P  W  T  G  G  I  F  L  V
1321 tgctccgttcgtctcttcacctctcatctgcttgcacgctgccatcggatccacgatagg 1380
     A  P  F  V  S  S  P  L  I  C  L  H  A  A  I  G  S  T  I  G
1381 gatgttagcagcactcagcattgccacaccatttgactccatctactttggcctgtgtgg 1440
     M  L  A  A  L  S  I  A  T  P  F  D  S  I  Y  F  G  L  C  G
1441 cttcaatagcactctggcctgtatcgctattggtggcatgttctacgtcatcacctggca 1500
     F  N  S  T  L  A  C  I  A  I  G  G  M  F  Y  V  I  T  W  Q
1501 gacgcacctgctcgccattgcctgtgccctgtttgcagcctacctgggtgctgccctggc 1560
     T  H  L  L  A  I  A  C  A  L  F  A  A  Y  L  G  A  A  L  A
1561 caacatgctgtctgtgtttggattaccaccctgcacctggccctctgcctctcagcgct 1620
     N  M  L  S  V  F  G  L  P  P  C  T  W  P  F  C  L  S  A  L
1621 caccttcctctcctcacaaccaacaaccctggcatctacaagctcccgctcagcaaagt 1680
     T  F  L  L  L  T  T  N  N  P  G  I  Y  K  L  P  L  S  K  V
1681 cacctacccagaggccaaccgcatctacttcctgtcccaggagaaaaacagaagggcatc 1740
     T  Y  P  E  A  N  R  I  Y  F  L  S  Q  E  K  N  R  R  A  S
1741 gatgataacaaagtaccaggcctacgacgtctcctaagctgccctttctaagctcgtctg 1800
     M  I  T  K  Y  Q  A  Y  D  V  S
1801 gtaaatgcagcctccagaacccaccctgcaccccaggctaaatctaagtcactcaaccac 1860
1861 ccttcttcctaatatgcgagtggctgctatttggagccctcaatgagatgtgcagcgctc 1920
1921 atcaaagccacgctagtttaaactaaatacccaccgatggctctttgggaactctctgtg 1980
1981 gggggaacttgctagtcacctctttcagttatttagtggcgcaaatcatgattccaggaa 2040
2041 atgttcattagcctgccatgcaagagggctgggttcactaatgagttccaactgtctcag 2100
2101 gccagtcttctccctaccccatccccaccctatccacacccaccccaccctcatctca 2160
2161 gcctgggtgttttgttgccaaggatttctttaccatgacaaggaaggactccaaagtcct 2220
2221 tccacctcagaagcatttattcttttccatatcctgtaaactaaccaactcagcatggcc 2280
2281 agcaagtaattattctgacttgagttacttatggaatggctctcagaggagagaggaaaa 2340
2341 tgttagtgccctgtggaagtttattcttagatatctgggagatgctctaaggcttgggac 2400
2401 cggacacctgaattcattgaaaatgccaatgtatcttctttgcaccatagaaatatttcc 2460
2461 agaaacatcatctggtaaggagtgacctttccaacatccaatcatagtgtaagcaaacg 2520
2521 tctttgcttgtgtaatattcaaaatcagtagtcattaaacttgaaatttgcaaaaaaaaa 2580
2581 aaaa  2584
```

*Figure 1.* (A) UT-A3 cDNA and amino acid sequence. (B) UT-A4 cDNA and amino acid sequence. These sequences have been submitted to GenBank. The accession numbers are: AF041788 (UT-A3), AF042167 (UT-A4).

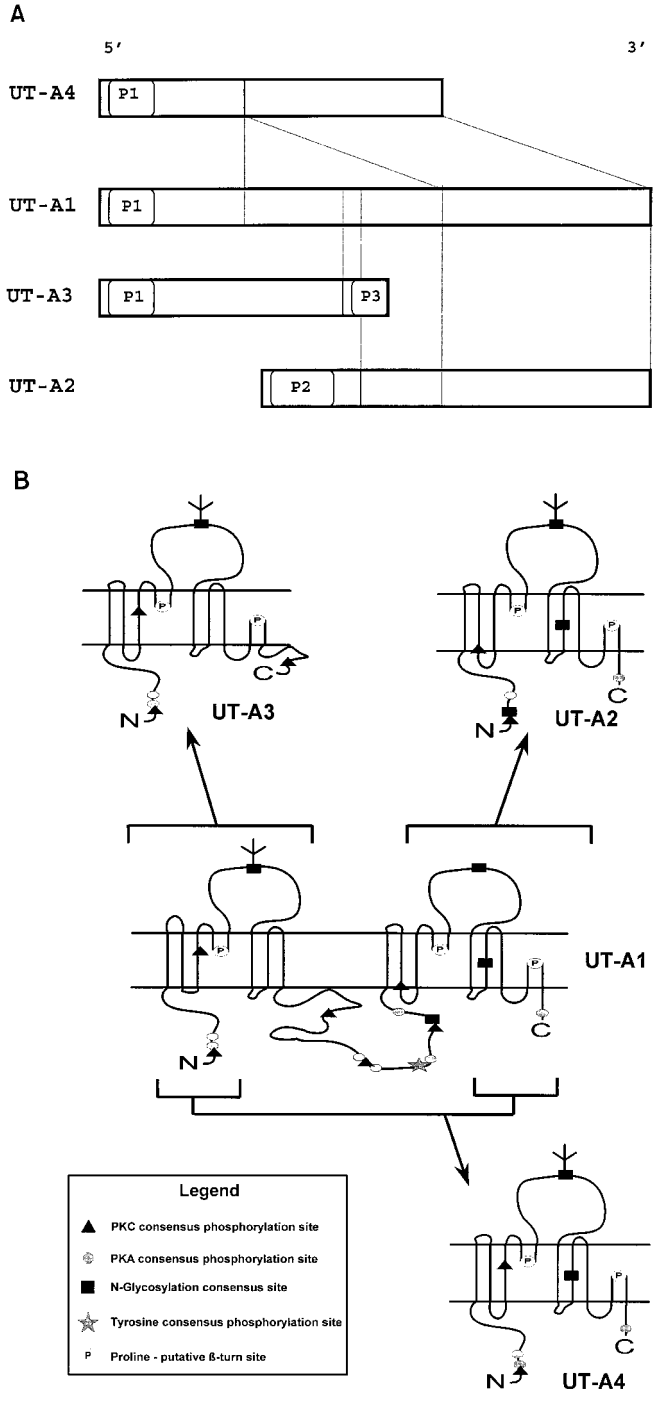


*Figure 2.* (A) Schematic illustration of the relationships between the cDNA of UT-A1, and the cDNA of the other isoforms UT-A2, UT-A3, and UT-A4. (B) Model of predicted membrane topology for UT-A3 and UT-A4 peptides and structural relationships with UT-A1 and UT-A2. (The UT-A1 and UT-A2 model are redrawn from reference (1).)

Phloretin-inhibitable urea transport was examined for UT-A3, UT-A4, and UT-A2, with or without forskolin, an activator of adenylate cyclase (Figure 4B). Forskolin induced a statistically significant increase in the transport activity of UT-A3 and UT-A4, 26% and 73%, respectively. As expected, forskolin did not increase the transport rate of UT-A2, which was slightly decreased (16%).

### *UT-A3 and UT-A4 mRNA Expression in Rat Tissues*

The expression of UT-A3 and UT-A4 mRNA was examined in multiple rat tissues by Northern hybridization, using three different DNA probes, spanning specific portions of the UT-A2, UT-A1, and UT-A3 cDNA (Figure 5). The position of the probes relative to specific cDNA sequences is illustrated in Figure 2. Probe P2, corresponding to a unique 5′ end cDNA sequence of UT-A2, hybridizes to a single transcript of 3.1 kb in the whole kidney, consistent with expression of UT-A2 limited to the kidney. Probe P1, corresponding to nucleotides 26 to 481, which are common to UT-A1, UT-A3, and UT-A4, hybridizes with transcripts of 4.0 kb and 2.1 kb in the kidney, consistent with the mRNA size of UT-A1 and UT-A3, respectively. There also is a weaker signal of 3.7 kb. The significance and specificity of the smaller signals (about 1.7 kb) appearing in most lanes is unclear at this time. Probe P3, corresponding to the unique 3′ UTR sequence of UT-A3, hybridizes with two transcripts of 3.7 kb and 2.1 kb in the kidney. The 2.1-kb transcript, identified with both probe P1 and probe P3, is consistent with expression of UT-A3 in the kidney. The nature of the 3.7-kb mRNA detected with probe P1 and probe P3 in the kidney is unclear at this time and needs further characterization. Among other tissues, only the testis expresses transcripts recognized by probe P3 with estimated size of 1.7 and 3.3 kb. Whether they represent shorter variants of the two transcripts detected by the same probe in the kidney remains to be established.

We could not detect any 2.5-kb mRNA, the size expected for UT-A4, in any tissue, suggesting that the abundance or stability of the UT-A4 transcript may be too low for detection by Northern hybridization. To detect expression of UT-A4 and to examine the distribution of UT-A3 and UT-A4 mRNA within regions of the kidney, we performed RT-PCR analysis of total RNA from renal cortex, outer medulla, and inner medulla (Figure 6). A sense primer corresponding to nucleotides 790 to 812 of UT-A3 cDNA and an antisense primer corresponding to nucleotides 1771 to 1790 of the 3′ UTR of UT-A3 were used to amplify a portion of UT-A3, and yielded a PCR product of the expected size of 1.0 kb in outer medulla (lane 1) and in inner medulla (lane 5), but not in cortex (lane 3). Because no unique sequence for UT-A4 is available, primers were designed to span segments of UT-A4 and of UT-A1 that could be distinguished by their size. Two PCR amplifications with nested primers detected 570 bp of UT-A4 in outer medulla (lane 2), and in inner medulla (lane 6), but not in cortex (lane 4). These same primers also detect a larger product of approximately 1.9 kb (lane 6) in inner medulla, but not in outer medulla or in cortex, consistent with expression of UT-A1 limited to the inner medulla, as shown previously (19,13). Thus, UT-A3 and UT-A4 are both expressed in the outer and inner medulla, with a relatively lower level of expression for UT-A4 mRNA.

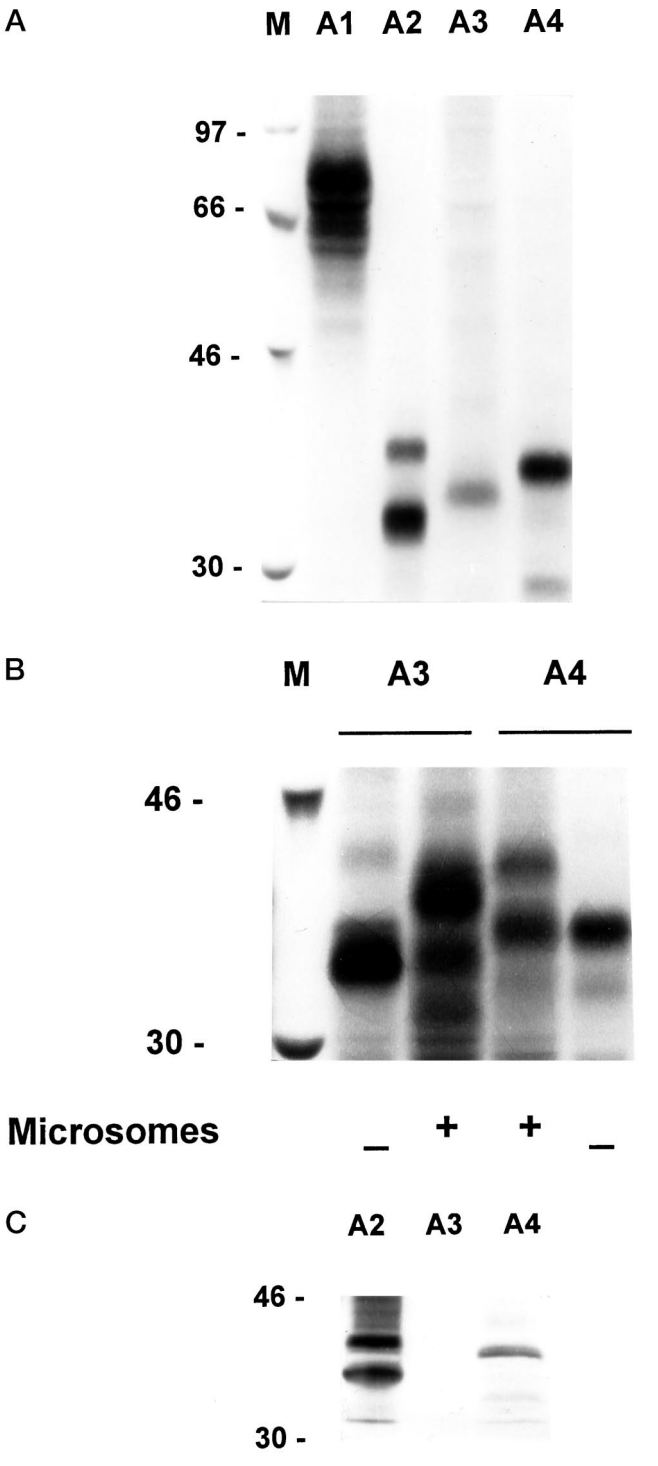


*Figure 3.* Analysis of *in vitro* translation products of the UT-A isoforms. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *in vitro* translation products of pcDNA3-UT-A1 (A1), pcDNA3-UT-A2 (A2), pcDNA3-UT-A3 (A3), and pcDNA3-UT-A4 (A4). Molecular size markers (M) are shown in the first lane. (B) *In vitro* translation products of pcDNA3-UT-A3 (A3) and pcDNA3-UT-A4 (A4) obtained in the presence (+) and in the absence (−) of pancreatic microsomes. Molecular size markers (M) are shown in the first lane. (C) Immunoblot of *in vitro* translation products of pcDNA3-UT-A2 (A2), pcDNA3-UT-A3 (A3), and pcDNA3-UT-A4 (A4), with a primary rabbit polyclonal antibody generated toward the carboxy terminus of the rat UT-A1/UT-A2 peptides (21).

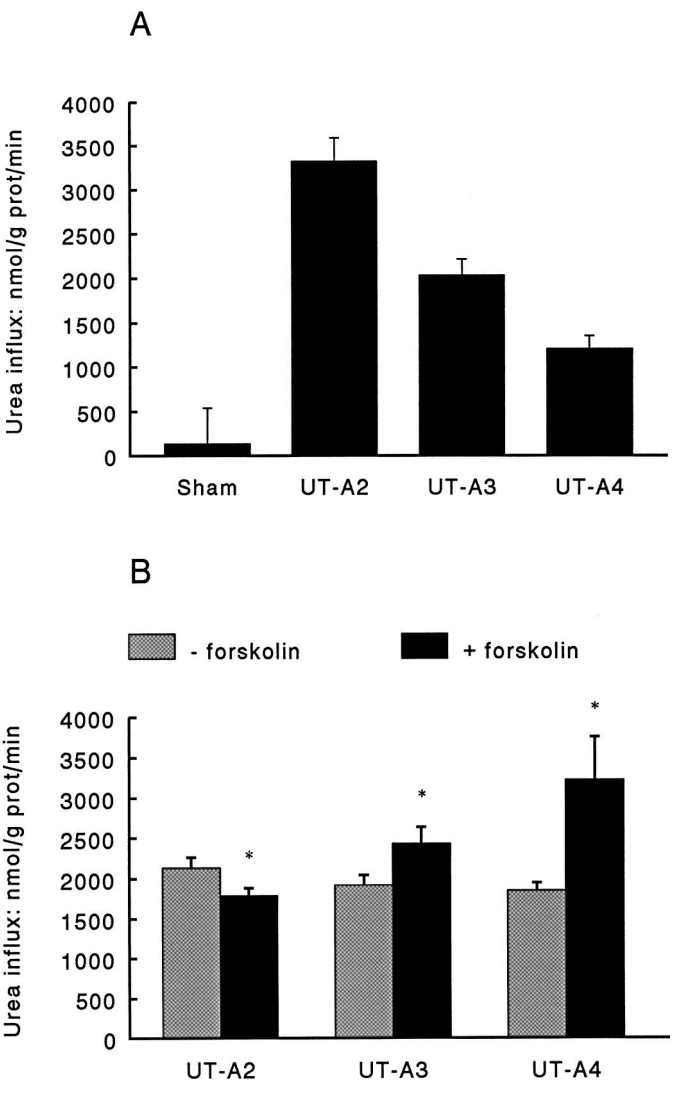


*Figure 4.* (A) Phloretin-inhibitable uptake of [$^{14}$C] urea in HEK-293 cells transiently transfected with pcDNA3-UT-A2, pcDNA3-UT-A3, pcDNA3-UT-A4, or pcDNA3 without insert (Sham), in the presence of 50 $\mu$M phloretin. (B) Effect of forskolin on phloretin-inhibitable urea uptake of HEK cells transiently transfected with pcDNA3-UT-A2, pcDNA3-UT-A3, and pcDNA3-UT-A4 (10 $\mu$M forskolin, 24 h preincubation). The unlabeled urea concentration in the uptake solution was 5 mM. Values are shown as mean ± SD ($n = 4$). *$P < 0.01$.

## Discussion

We have identified two new isoforms of the UT-A urea transporter: UT-A3 and UT-A4. Analysis of the homology of UT-A3 and UT-A4 cDNA sequences with those of UT-A1 and UT-A2 shows that each of the two new isoforms corresponds to different portions of UT-A1. Thus, it appears likely that UT-A3 and UT-A4 as well as UT-A1 and UT-A2 may be encoded by a single gene. Interestingly, the structural models proposed for UT-A3 and UT-A4 show symmetric configurations similar to UT-A2, and come very close to the model predicted for the erythrocyte urea transporter UT-B (UT11/UT3) (14), to which UT-A3 and UT-A4 are 61% homologous. Another interesting feature of the new isoforms is that both

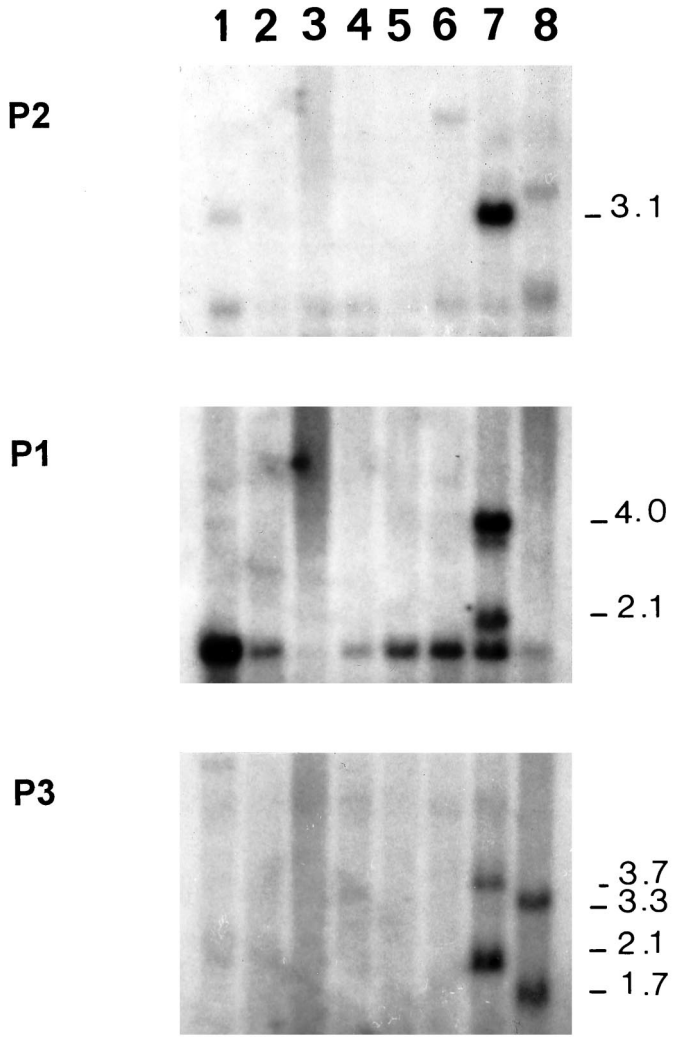


*Figure 5.* Northern hybridization of rat multiple tissue blot. Lanes are as follows: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. (Top Panel) Membrane hybridized with DNA probe P2, (nucleotides 63/492 of UT-A2 cDNA; Figure 2A). (Middle Panel) Membrane hybridized with probe P1 (nucleotides 26/481 of UT-A1, UT-A3, and UT-A4 cDNA; Figure 2A). (Bottom Panel) Membrane hybridized with probe P3 (nucleotides 1746/1986 of UT-A3 cDNA; Figure 2A). (The faint bands of 3.3 and 1.7 kb seen in lane 8 of the top panel may be due to incomplete elimination of the signal from previous hybridization of the same membrane with probe P3. There is not sufficient homology between probes P2 and P3 to expect cross-hybridization.)

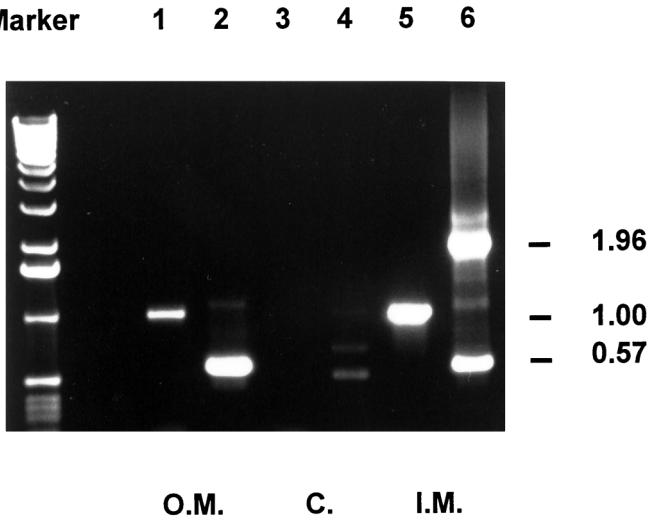


*Figure 6.* Renal expression of UT-A3 and UT-A4 detected by reverse transcription-PCR, using RNA from rat kidney outer medulla (lanes 1 and 2), cortex (lanes 3 and 4), and inner medulla (lanes 5 and 6). The size of the specific amplification products (indicated in kilobases) is shown on the left, and the first lane contains DNA size markers (M, 1-kb ladder). A single amplification was used for UT-A3, spanning nucleotides 790 to 1790 of UT-A3 cDNA, resulting in a 1.0-kb product (lanes 1, 3, and 5). The amplification to detect UT-A4, spanning nucleotides 1008 to 2964 of UT-A1 cDNA, yields two products: the smaller, about 0.57 kb, representing UT-A4 (lanes 2, 4, and 6) and the larger, about 1.96 kb, resulting from UT-A1 (lane 6).

UT-A3 and UT-A4 peptides include all of the following three amino acid motifs: KWDLPFTLPFN, ATGHYNLFFP, and SSPLICLHAAIGS, which are present as intra-protein tandem repeats within each of the two UT-A1 halves (UT-A1a and UT-A1b), and which, as we have previously noted (1), are found in all urea transporters, including UT-B. Thus, these may represent highly conserved "signature sequences" in the urea transporter family.

UT-A3 and UT-A4 are functionally active urea transporters, and display levels of phloretin-inhibitable urea uptake in the range of those reported previously for the other two known isoforms UT-A1 and UT-A2. In addition, they appear to be susceptible to regulation via a cAMP-dependent pathway based on the stimulatory effect of forskolin on their urea uptake activity. Several consensus sequences for protein kinase A-dependent phosphorylation sites are present in both UT-A3 and UT-A4, of which those located at the intracytoplasmic amino-terminal and carboxy-terminal domains may be important for *in vivo* regulation of their functional activity as urea transporters. Our observations support the possibility that the activity of UT-A3 and UT-A4 may be regulated by vasopressin, through activation of adenyl cyclase and generation of cAMP.

We do not have a precise explanation of the reason why the *in vitro* translation products of UT-A3 and UT-A4 are lower than their predicted molecular weight. In our hands, the *in vitro*-translated products of UT-A1 and UT-A2 also show a lower molecular weight than predicted, similar to observations by Shayakul *et al.* (13), who, using the same reagent system, reported a molecular weight of 76 kD for the translated product of the rat UT-A1, while the predicted molecular weight is 95 kD.

Analysis of the expression of the different UT-A mRNA isoforms in rat tissue confirms that the UT-A transporters are localized primarily within the kidney, but indicates that expression of some UT-A isoforms also occurs in the testis, which was never reported previously; expression of an isoform of UT-B, the erythrocyte urea transporter, has been detected in the testis (16,24). In addition, our findings show that the UT-A mRNA expressed in the testis are smaller than their renal

counterparts, perhaps as a result of tissue-specific regulatory mechanisms. Our observations also point to the existence of another, as yet uncharacterized UT-A isoform of approximately 3.7 kb in the kidney. The intrarenal distribution of UT-A3 and UT-A4 mRNA expression was examined by RT-PCR, confirming the presence of both mRNA in the rat kidney. The pattern of expression of UT-A3 and UT-A4 indicates that their mRNA is expressed in outer and inner medulla but not in cortex. Thus, the distribution of UT-A3 and UT-A4 is similar to that of UT-A1 and UT-A2, for which expression appears confined to the medullary region (17–19).

In conclusion, we have identified UT-A3 and UT-A4 as two new isoforms of the UT-A urea transporter, which are expressed in the renal inner medulla. They are functionally active urea transporters and their activity appears to be stimulated by cAMP-dependent pathways, suggesting that they may be regulated by vasopressin. The high homology of these new isoforms and the previously identified UT-A transporters support their origin from a single gene. Our findings indicate that other, as yet uncharacterized, related forms of this family of transporters may be expressed in the testis. Additional studies will be necessary to understand the regulation of the expression and function of multiple urea transporters in the kidney and in other organs.

## Acknowledgments

This work was supported in part by National Institutes of Health Grants PO1-DK50268, R01-DK41707, and R37HL28674. We thank Dr. William E. Mitch for critical review of the manuscript. We also thank Sharon D. Langley for her invaluable help in sequencing our samples.

## References

1. Sands JM, Timmer RT, Gunn RB: Urea transporters in kidney and erythrocytes. *Am J Physiol* 273: F321–F339, 1997
2. Sands JM, Nonoguchi H, Knepper MA: Vasopressin effects on urea and $H_2O$ transport in inner medullary collecting duct subsegments. *Am J Physiol* 253: F823–F832, 1987
3. Chou C, Knepper MA: Inhibition of urea transport in inner medullary collecting duct by phloretin and urea analogues. *Am J Physiol* 257: F359–F365, 1989
4. Chou C, Sands JM, Nonoguchi H, Knepper MA: Concentration dependence of urea and thiourea transport pathway in rat inner medullary collecting duct. *Am J Physiol* 258: F486–F494, 1990
5. Chou C, Sands JM, Nonoguchi H, Knepper MA: Urea-gradient associated fluid absorption with $\sigma_{urea}=1$ in rat terminal collecting duct. *Am J Physiol* 258: F1173–F1180, 1990
6. Kishore BK, Terris J, Fernandez-Llama P, Knepper MA: Ultramicro-determination of vasopressin-regulated renal urea transporter protein in microdissected renal tubules. *Am J Physiol* 272: F531–F537, 1997
7. Knepper MA, Sands JM, Chou C: Independence of urea and water transport in rat inner medullary collecting duct. *Am J Physiol* 256: F610–F621, 1989
8. Sands JM, Knepper MA: Urea permeability of mammalian inner medullary collecting duct system and papillary surface epithelium. *J Clin Invest* 79: 138–147, 1987
9. Star RA, Nonoguchi H, Balaban R, Knepper MA: Calcium and cyclic adenosine monophosphate as second messengers for vasopressin in the rat inner medullary collecting duct. *J Clin Invest* 81: 1879–1888, 1988
10. You G, Smith CP, Kanai Y, Lee W-S, Stelzner M, Hediger MA: Cloning and characterization of the vasopressin-regulated urea transporter. *Nature* 365: 844–847, 1993
11. Smith CP, Lee W-S, Martial S, Knepper MA, You G, Sands JM, Hediger MA: Cloning and regulation of expression of the rat kidney urea transporter (rUT2). *J Clin Invest* 96: 1556–1563, 1995
12. Olivès B, Martial S, Mattei MG, Matassi G, Rousselet G, Ripoche P, Cartron JP, Bailly P: Molecular characterization of a new urea transporter in the human kidney. *FEBS Lett* 386: 156–160, 1996
13. Shayakul C, Steel A, Hediger MA: Molecular cloning and characterization of the vasopressin-regulated urea transporter of rat kidney collecting ducts. *J Clin Invest* 98: 2580–2587, 1996
14. Olives B, Neau P, Bailly P, Hediger MA, Rousselet G, Cartron JP, Ripoche P: Cloning and functional expression of a urea transporter from human bone marrow cells. *J Biol Chem* 269: 31649–31652, 1994
15. Couriaud C, Ripoche P, Rousselet G: Cloning and functional characterization of a rat urea transporter: Expression in the brain. *Biochim Biophys Acta* 1309: 197–199, 1996
16. Tsukaguchi H, Shayakul C, Berger UV, Tokui T, Brown D, Hediger MA: Cloning and characterization of the urea transporter UT3: Localization in rat kidney and testis. *J Clin Invest* 99: 1506–1515, 1997
17. Bagnasco SM, Jacobs JD, Timmer RT, Klein JD, Froehlich O, Gunn RB, Sands JM: Immunolocalization of urea transporter proteins in human and rat kidney [Abstract]. *FASEB J* 11: A24, 1997
18. Nielsen S, Terris J, Smith CP, Hediger MA, Ecelbarger CA, Knepper MA: Cellular and subcellular localization of the vasopressin-regulated urea transporter in rat kidney. *Proc Natl Acad Sci USA* 93: 5495–5500, 1996
19. Shayakul C, Knepper MA, Smith CP, DiGiovanni SR, Hediger MA: Segmental localization of urea transporter mRNAs in rat kidney. *Am J Physiol* 272: F654–F660, 1997
20. Star RA: Apical membrane limits urea permeation across the rat inner medullary collecting duct. *J Clin Invest* 86: 1172–1178, 1990
21. Naruse M, Klein JD, Ashkar ZM, Jacobs JD, Sands JM: Glucocorticoids downregulate the rat vasopressin-regulated urea transporter in rat terminal inner medullary collecting ducts. *J Am Soc Nephrol* 8: 517–523, 1997
22. Frohman MA, Dush MK, Martin GR: Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 85: 8998–9002, 1988
23. Timmer RT, Gunn RB, Froehlich O, Bagnasco SM, Klein JD, Sands JM: Heterologous expression and characterization of the 2.9 kb isoform of the rat renal urea transporter [Abstract]. *Biophys J* 72: A413, 1997
24. Promeneur D, Rousselet G, Bankir L, Bailly P, Cartron JP, Ripoche P, Trinh-Trang-Tan MM: Evidence for distinct vascular and tubular urea transporters in the rat kidney. *J Am Soc Nephrol* 7: 852–860, 1996