Tissue distribution of UT-A and UT-B mRNA and protein in rat

John J. Doran, Janet D. Klein, Young Hee Kim, Tekla D. Smith, Shelley D. Kozlowski, Robert B. Gunn, and Jeff M. Sands

Renal Division, Department of Medicine and Department of Physiology, Emory University School of Medicine, Atlanta, Georgia

Submitted 1 June 2004; accepted in final form 19 December 2005

Mammalian urea transporters are facilitated membrane transport proteins belonging to two families, UT-A and UT-B. They are best known for their role of maintaining the renal inner medullary urinary concentrating gradient. Urea transporters have also been identified in tissues not typically associated with urea metabolism. The purpose of this study was to survey the major organs in rat to determine the distribution of UT-A and UT-B mRNA transcripts and protein forms and determine their cellular localization. Five kidney subregions and 17 extrarenal tissues were screened by Northern blot analysis using two UT-A and three UT-B probes and by Western blot analysis using polyclonal COOH-terminal UT-A and UT-B antibodies. Immunohistochemistry was performed on 16 extrarenal tissues using the same antibodies. In kidney, we detected mRNA transcripts and protein bands consistent with previously-identified UT-A and UT-B isoforms, as well as novel forms. We found that UT-A mRNA and protein are widely expressed in extrarenal tissues in various forms that are different from the known isoforms. We determined the cellular localization of UT-A and UT-B in these tissues. We found that both UT-A and UT-B are ubiquitously expressed as numerous tissue-specific mRNA transcripts and protein forms that are localized to cell membranes, cytoplasm, or nuclei.

urea transporter; Northern blot analysis; Western blot analysis; immunohistochemistry

UREA TRANSPORTERS (UT) are facilitated membrane transport proteins that move urea across cell membranes. In mammals, they arise from two genes, UT-A (Slc14a2) (1, 26) and UT-B (Slc14a4a) (24), that are in tandem on chromosome 18 (11) and have a fairly high degree of inter- and intragene homology. The UT-A gene is larger (1, 10, 26) and gives rise to at least nine isoforms (splice variants) due to two promoter sites (alternative transcriptional start sites), differential splicing and alternative 3’ untranslated regions (UTRs) (2, 17, 26, 34, 37, 38, 53). These isoforms, designated UT-A1, UT-A1b, UT-A2, UT-A2b, UT-A3, UT-A3b, UT-A4, UT-A5, and UT-A6, predict six UT-A proteins of 929, 397, 460, 466, 323, and 235 amino acids, respectively. Most of the UT-A isoforms have been cloned from kidney (2, 13, 17, 34, 35, 37, 53), except for UT-A5 [mouse testis (12)] and UT-A6 [human colon (38)]. In the mouse kidney, UT-A1 and/or UT-A3 are major contributors to the urinary concentrating mechanism, whereas UT-A2 has a minor role under physiologic conditions. Under basal conditions, the UT-A1/A3 null mouse has a severe concentrating defect (8), whereas the UT-A2 null mouse has a minor defect that is apparent when it is water-deprived and on a low protein diet (47).

A number of extrarenal tissues have been shown to express UT-A protein and/or mRNA, including rat heart (7), liver (20), colon (53), and mouse testis, heart, liver, brain (13), and colon (40). The cellular location of UT-As have been identified in cochlear epithelial and nonepithelial cells (21), colonic epithelial cells (40), and in testis [where UT-A5 is in the outer layer of seminiferous tubules (12) and UT-A1 or -A3 is in Sertoli cells], Leydig cells, and spermatid residual bodies (9). These localization studies have helped generate hypotheses regarding the physiologic roles of UT-A, although regulatory studies have not been performed in these tissues. Conversely, regulation of UT-A has been demonstrated in heart (in uremia, hypertension, and heart failure) (7) and liver (in uremia) (20), although the cellular localization of UT-A in these tissues is unknown.

In human, UT-B was cloned from bone marrow (HUT111) (30), reticuloocytes (HUT111A) (36), and colon (15) and was originally called the erythrocyte urea transporter because it is expressed on red blood cells as a minor blood group antigen, Kidd or Jk (29). Two UT-B mRNAs (2.0 and 4.4 kb) arise from alternative polyadenylation sites (24) and predict a single, 389 amino acid, 45-kDa glycoprotein. In rodent, UT-B was cloned from kidney (5, 46) and two mRNAs of 2.0 kb (mouse only) and ~4.0 kb (3, 5, 10, 33, 46, 52) predict a 384 amino acid, 37- to 51-kDa protein (44, 52). The UT-B null mouse has a urea selective concentrating defect (52), which is thought to involve UT-B in both the vasa recta and erythrocytes.

UT-B mRNA or protein has been detected in a variety of rodent extrarenal organs including bone marrow, brain, cochlea, spleen, testis, thymus, heart, aorta, mesenteric artery, lung, liver, duodenum, colon, skeletal muscle, ureter, urethra, and bladder (3, 5, 14, 21, 33, 39, 40, 44–46, 49). Many human organs express UT-B as a variety of mRNAs (2.0, 3.0, 3.6, 4.4, and 4.5 kb), including brain, prostate, bladder, heart, skeletal muscle, colon, intestine, spleen, pancreas, and thymus (28, 30, 32).

The cellular localization of extrarenal UT-B is better studied than UT-A. Rodent extrarenal UT-B is in epithelium of ureter, urethra, and bladder (23, 39). It has also been localized in numerous cell types in brain (3, 23) and cochlea (21), Sertoli cells in testis (9, 46), colonic epithelial cells (human) (15), and bovine ruminal epithelium from which a larger UT-B variant (bUT-B2) was cloned (41). The regulation of UT-B has been
studied in a few organs in lamb (25) and rodent. In rat, it is upregulated in brain by uremia (14) and neurotoxins (3), but in uroepithelium the data is not consistent (23, 39).

In the present study, we determined the tissue distribution of UT-A and UT-B in rat by screening with Northern and Western blot analysis and immunohistochemistry. We report novel UT-A and UT-B transcripts in kidney and extrarenal tissues and show that both are widely expressed among many tissues. We propose that UT-A and UT-B play tissue-specific roles where they transport urea, or perhaps a yet-unidentified substrate, for purposes that remain to be elucidated.

METHODS

Tissue Preparation

Whole organs were removed from male Sprague-Dawley rats (~250 gm; National Cancer Institute, Frederick, MD). Kidneys were dissected into cortex, outer medulla (OM), and inner medulla (IM); the latter was further divided into three equal parts along its length: base, mid, and tip. Brain was dissected into cerebrum and cerebellum. Gastrointestinal organs were manually evacuated and washed clean with PBS. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee.

RNA isolation and purification. Tissues were immediately frozen in liquid nitrogen, and then directly homogenized in Trizol reagent (Life Technologies, Rockville, MD) according to the manufacturer’s protocol. The RNA was treated with DNase (1 U/200 μg RNA) and then proteinase K (0.75 U/200 μg RNA), extracted three times with phenol/chloroform (5:1, pH 4.5), precipitated with ethanol/ammonium acetate (2.5:0.5, vol/vol), and dissolved in water. For some tissues, a single round of poly(A) RNA purification was performed according to the manufacturer’s directions with the use of one of the following kits: Poly(A) Pure (Ambion, Austin TX), Oligotex (Qiagen, Valencia CA), or PolyATract (Promega, Madison WI).

Protein isolation and Western blot analysis. Tissues were placed into an ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6, 1 μg/ml leupeptin, and 2 mg/ml PMSF) and homogenized with an Omni motorized tissue grinder or with glass homogenizers. SDS was added to a final concentration of 1%, and the samples were sheared with a 25-gauge needle. Homogenates were centrifuged at 8,000 g for 15 min, and the protein in the supernatant fractions was measured by a modified Lowry method (DC Protein Assay Kit; Bio-Rad, Hercules, CA). Proteins (15 μg/lane) were size separated by SDS-PAGE by using 10% gels then electroblotted to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS: 20 mM Tris HCl, 0.5 M NaCl, pH 7.5) at room temperature for 1 h and then incubated with primary antibody overnight at 4°C. Primary antibodies were polyclonal COOH-terminal antibodies to UT-A (18) and UT-B (44). The COOH-terminal antibody to UT-A will detect UT-A1, UT-A2, and UT-A4, but not UT-A3, UT-A5, or UT-A6 (Fig. 1). The COOH-terminal UT-B antibody detects the (only known) UT-B protein (Fig. 2). Blots were washed three times in TBS with 0.5% Tween-20 (TBS/Tween), and then incubated with horseradish peroxidase-linked goat anti-rabbit IgG at a dilution of 1:4,000 to 1:5,000 (Amersham, Arlington Heights, IL) for 2 h at room temperature. If infrared detection was to be used, this secondary antibody was replaced with Alexa Fluor 680-linked anti-rabbit IgG (Molecular Probes, Eugene, OR). Blots were washed two times with TBS/Tween, and then the bound secondary antibody was visualized by using chemiluminescence (ECL kit, Amersham) or infrared detection with the Licor Odyssey protein analysis system. Antibody competition studies were performed for UT-A in which the
primary antibody was preincubated with the immunizing peptide (0.2 μg/ml) (27). In all cases, parallel gels were stained with Coomassie blue and showed uniformity of loading.

Reticulocyte mRNA Isolation from Darbepoetin-Treated Rats

Male Sprague-Dawley rats weighing 250 g were given a single subcutaneous dose (15 μg) of darbepoetin to stimulate erythropoiesis. Four days later, the rats were killed, and 10 ml of whole arterial blood was collected from the aorta into EDTA-containing tubes. Reticulocyte layers were isolated by a continuous density gradient centrifugation method modified from a published protocol for human reticulocyte isolation (31). One milliliter of rat blood was added to Percoll columns, and centrifuged at 15,000 rpm at 4°C for 40 min, and the clear reticulocyte layers were pooled and divided into tubes of 7.5-ml aliquots. PBS (15 ml) was added to each tube and centrifuged at 5,000 rpm for 5 min at 4°C. This step was repeated twice, and the (less dense, upper) reticulocyte layer was identified and transferred into a 15-ml tube containing Trizol. Total mRNA was isolated as described in RNA isolation and purification. Human and rabbit bone marrow reticulocyte mRNA was a kind gift from Dr. Richard T. Timmer (Emory University).

Templates for UT-A RNA Probes

Rat UT-A4 cDNA and UT-A3 cDNA were each cloned into pcDNA3 (which has a flanking SP6 promoter site) and was linearized using BamHI. The resultant UT-A Probe 1 includes the last 389 nt of the COOH-terminal end of the UT-A3 coding region and will detect UT-A1, UT-A1b, UT-A3, UT-A3b, UT-A5, and UT-A6 with 100% (A1), 99% (A3), 91% (A5, mouse sequence, GenBank accession no. AF258601), and 84% (A6, human sequence, GenBank accession no. AK074236) identity to the target regions, respectively (Fig. 1). UT-A Probe 2 includes the last 408 nt of the UT-A4 coding region and will detect UT-A1, UT-A1b, UT-A2, UT-A2b, and UT-A4, all with 100% identity to the target region (Fig. 1). Due to a large degree of homology within UT-A1, UT-A Probe 1 and UT-A Probe 2 are 64% homologous to each other. These templates were commercially sequenced to verify their identity. Templates for GAPDH (used as a control for tissues that were negative by UT-A or UT-B probes) and the molecular weight marker probes were purchased as linearized pTRIPLEscript plasmids: pTRI-GAPDH rat and pTRI-MMP (Ambion).

Three UT-B probes (Fig. 2) were used in these studies, not by a priori design, but because the data are a compilation of studies that used different probes. We obtained the same results with any of the probes, although the actual UT-B probe used for a particular blot is specified in the figure legends. Full-length probes were chosen to maximize sensitivity (having more incorporated radiolabeled nucleotide triphosphates per probe), albeit sacrificing specificity of distinguishing a smaller transcript. A 1,000-bp probe can potentially have five times the sensitivity of a 200-bp probe for detecting a 200-bp target, and the unhybridized (dangling) region contribute to the overall signal.

Templates for UT-B Probes

Templates were synthesized by PCR using either of the following methods: 1) the complementary PCR primer included the SP6 promoter sequence at its 5’ end so the PCR products were used directly without cloning; or 2) PCR products were cloned into pST-Blue-1 (Novagen, Madison, WI) which has 3’ dU overhangs (for directly cloning Tag-synthesized PCR products) and a SP6 promoter region flanking the insert site. The templates were commercially sequenced to verify their identity. Rat kidney RNA (2 μg) was reverse transcribed using avian myeloblastosis virus or Moloney murine leukemia virus reverse transcriptase to cDNA, and then amplified by PCR with DNA polymerase (Advantage 2; Clontech, Palo Alto, CA or Master-Taq, Eppendorf, Westbury, NY) for 35–40 cycles (30–45 s at 95°C, 30–45 s at 50–55°C, 2–3 min at 68–72°C). In both cases, the PCR products were size separated by electrophoresis in tris-acetate-EDTA buffer and gel isolated. Primer pairs for the UT-B probes included [designed to UT-3 sequence, GenBank accession no. X98399 (19)] UT-B 868-bp probe: (5’-ACAAGCGGCGTTGCTCCAGTT-3’) and (5’-CAGGTACAGCTGGAGTCAA-3’); UT-B 975-bp probe: (5’-ACAAGCGGCGTTGCTCCAGTT-3’) and (5’-AGATGCCGTTCTCCCTCGAGATA-3’); and UT-B 1,111-bp probe: (5’-ATGGGAAGATATCCACTGATGG-3’) and (5’-AGATGCGCGTTCTCCTCGAGATA-3’).

Probe Synthesis

Probes were synthesized by in vitro transcription using SP6 (incorporating [α-32P]UTP, 3,000–6,000 Ci/ml/m), purified through P-30 polyacrylamide gel Micro Bio-Spin columns (Bio-Rad) and combined (0.2 μl) with water (500 μl) and Opti-Fluor (2 ml; Perkin-Elmer, Shelton, CT) for scintillation counting. Unlabeled probes were also synthesized and size separated by electrophoresis to verify the expected size.

Northern Blot Analysis

Molecular mass markers (diluted to 0.05 μg/ lane) and each sample of total (5–10 μg) or poly(A) (0.6–2 μg) RNA were mixed with loading buffer, size separated by electrophoresis (4–6 V/cm) on 1% agarose gels in glyoxal buffer (Ambion), blotted to positively-charged nylon membranes and cross-linked with UV light (120 mJ for 30 s) (GS Gene Linker; Bio-Rad). The amount of loaded RNA varied as it was optimized empirically (based on final autoradiograms) for each tissue. If the autoradiograms showed nonspecific staining of the 18S or 28S ribosomal RNA bands (that could potentially obscure a UT band), the blots were repeated using poly(A) RNA. For other tissues, the blots were repeated with poly(A) RNA in an attempt to augment faint bands. Three membranes (blots) were prepared for each tissue and probed with each of the three primary probes (UT-A Probe 1, UT-A Probe 2, and UT-B Probe). This obviated any possibility that a UT band would be seen from residual signal. To verify the results, each tissue-probe combination was done at least three times so there were at least nine separate membranes (blots) for each tissue. Membranes were prehybridized using ZipHyb or UltraHyb (Ambion, 50% formamide) for 30 min at 68°C and then hybridized for 2 h (ZipHyb) or 15 h (UltraHyb) at 68°C (10 6 cpm/ml). Membranes were washed twice with low-stringency wash buffer (Ambion) at ~40°C for 5 min, and twice with high-stringency wash buffer (Ambion) at 68°C for 15 min. Additional washings at high stringency were done as needed to optimize band clarity. Autoradiograms were obtained by exposing membranes to film (BioMax MS or X-Omat, Kodak) with (~80°C) or without (25°C) intensifying screens, for 1 h to 5 days. In most cases, the same membrane required a number of different autoradiograms to optimize the clarity of a particular tissue (lane) and/or band (lane). After satisfactory images were obtained from UT-A Probe 1, UT-A Probe 2, or UT-B probe, each membrane was next probed with the GAPDH probe as described above. GAPDH was used to demonstrate mRNA integrity and successful blotting (as was the marker probe) in the cases where no UT bands were detected. If a residual UT-A or UT-B band fell within ~0.5 kb of GAPDH (1.4 kb), the membrane was washed [high-stringency wash buffer (Ambion) at 70°C for up to 60 min] or left to decay (>3 mo) before probing with the GAPDH probe. All tissues showed 1.4-kb bands from the GAPDH probe; however, because this study was not designed for quantitative mRNA comparisons, the GAPDH bands are not shown in the figures. Each membrane was probed a third time using the marker probe as described above.
Molecule Analysis, PCR Primer Design, and Melting Temperature Calculation

All molecules and PCR primers were designed and/or analyzed using Clone Manager v7.01 (Science and Educational Software, Durham, NC). Band sizes for Northern blot analysis were calculated with computer spreadsheets (Excel; Microsoft, Redmond, WA) by using a standard plot of marker size vs. migration distance for each lane, which was then used to calculate the mRNA size by its migration distance (6). Autoradiograms were digitized, stored as tiff images, and edited using Microsoft PowerPoint to optimize each lane for clarity. In all cases, the figures are shown with the least possible manipulation from the original autoradiogram. Melting temperature (Tm) was calculated according to the formula:

\[ Tm = 78 + 16.6 \log_{10} \left( \frac{[Na^+]_i}(1.0 + 0.7 [Na^+]) + 0.41(1+[G+C]) \right) - 500/n - P - F \]

where \( n \) is the duplex length, \( P \) is the temperature correction for %base mismatches (1°C/%mismatch) and \( F = (0.35°C/1% \text{ formamide}) \).

Immunohistochemistry

Organs were perfusion-fixed with 4% paraformaldehyde (PFA) in live, isofluorane-anesthetized rats. After being perfused with fixative for 5–7 min, organs were removed and transverse thick slices (2–3 mm) were placed in the 4% PFA overnight at 4°C. Tissues were then placed in histology cassettes and washed successively with fresh 4% PFA (2 h, 4°C), PBS (3 × 10 min), 50 mM NH₄Cl/PBS (1 h), PBS (3 × 10 min), 70, 96, and 98% ethanol (EtOH) (2 h each), and xylene (overnight). Tissues were transferred to pure paraffin at 56°C for 2 h and then embedded in paraffin.

Tissues were sliced to 2-μm thickness on a microtome and mounted on glass slides. After removing paraffin with xylene and washing 2 times with EtOH, endogenous peroxidase activity was blocked with 0.35% H₂O₂ in methanol for 30 min. After washing with 96% EtOH, 70% EtOH, and ddH₂O₂, target retrieval was accomplished by microwaving samples with 10 mM Tris, 0.5 mM EGTA buffer (full power until boiling followed by soft boiling at 60% power for 5 min). To prevent nonspecific antibody binding, sections were further blocked with 50 mM NH₄Cl in PBS (30 min) followed by 3 × 10 min incubation with 1% BSA, 0.2% gelatin, and 0.05% saponin in PBS. Tissues were then incubated with primary antibody, anti-UT-A11 (1:4,000 dilution) or anti-UT-B1 (1:2,000 dilution), in 0.1% BSA, 0.3% Triton X-100 in PBS overnight at 4°C in a humidified chamber. After equilibrating back to room temperature, sections were washed free of primary antibody with the 0.1% BSA/0.2% gelatin/0.05% saponin solution (3 × 10 min) and then incubated with horseradish peroxidase-labeled secondary goat anti-Rabbit IgG (DAKO, Carpinteria, CA) in the BSA/Triton diluent. For antibody competition studies, the primary antibody was preincubated with the immunizing peptide, and then adjacent tissue slices were probed using the preabsorbed antibody. Positive staining was visualized using a diaminobenzidine dye (brown). Cell nuclei were also counterstained with hematoxylin (blue).

RESULTS

UT-A in Kidney

Northern blot analysis. UT-A Probe 1 (targets UT-A1/A3/A5/A6) detects 2.1 (UT-A3b)-, 3.7 (UT-A3b)-, and 4.1-kb (UT-A1) bands in IM and 3.1-kb bands in OM and (faintly) in IM base (Fig. 3A). UT-A Probe 2 (targets UT-A1/A2/A4) detects a 4.1-kb band (UT-A1) in IM and a 3.1-kb band in OM and IM base (Fig. 3B). Bands with sizes and/or distribution that did not correspond to known isoforms were of 2.7, 3.2, and 3.6 kb in IM tip and IM mid, and of 1.5, 2.7, and 3.1 kb in cortex.

Western blot analysis. The COOH-terminal UT-A antibody detects a 117-kDa band in IM tip and IM mid and a 97-kDa band (UT-A1) in IM and OM (Fig. 3C). Two bands of 39 and 55 kDa (UT-A2) are detected in OM (as doublets) and as singlets in cortex. In IM tip and IM mid, the 39-kDa band is faintly seen, and a group of bands ~55 kDa is faintly seen in IM base.

UT-A in Extrarenal Tissues

UT-A Probe 2 detects bands in all extrarenal tissues (Figs. 4 and 5), whereas UT-A Probe 1 detects bands in only brain and testis (Fig. 4). The UT-A antibody detects bands in all tissues (Figs. 4 and 5).

Brain: Northern blot analysis. UT-A Probe 1 detects 1.8-, 3.8-, and 5.2-kb bands in cerebrum and the latter two, in cerebellum (Fig. 4A). UT-A Probe 2 detects bands of 1.3, 1.8, 2.7, 4.9, and 5.1 kb in both cerebrum and cerebellum (Fig. 4B).
Only the 1.8- and ~5.0-kb bands are detected by both probes and could possibly be the same transcripts, while the other bands are unique to one probe or the other.

**Brain:** **Western blot analysis.** The COOH-terminal UT-A antibody detects bands of 55, 61, 75, 87, and 120 kDa in cerebrum. The same bands are detected in cerebellum except the 120-kDa band. In both cerebrum and cerebellum the 55-kDa band has the greatest intensity, whereas the others are faint (Fig. 4C).

**Testis:** **Northern blot analysis.** UT-A Probe 1 detects diffuse ~1.6-kb (UT-A5) and 2.3-kb bands (Fig. 4D), and UT-A Probe 2 detects diffuse 1.5-, 2.7-, and 2.9-kb bands (Fig. 4E).

**Testis:** **Western blot analysis.** The COOH-terminal UT-A antibody detects a group of bands between 54 and 60 kDa and discrete bands at 80, 85, 98, and 113 kDa (Fig. 4F).

**Other extrarenal tissues:** **Northern blot analysis.** In the remaining tissues, UT-A Probe 2 (but not UT-A Probe 1) detects bands. In Fig. 5, A and C (except colon) the samples are total RNA, and in Fig. 5B (and colon) are poly(A) RNA. The smallest band of 1.5 kb is detected in ovary, lung, skeletal muscle, bladder (Fig. 5A), prostate, liver (Fig. 5B), stomach, and colon (Fig. 5C). The intensity of the 1.5-kb band is less than any of the larger bands in a given tissue. All of the tissues have a 2.7-kb and/or a 3.1-kb band, and the intensities of these varied among the tissues. The 2.7-kb band is found in ovary, spleen, lung, skeletal muscle, bladder (Fig. 5A), heart (Fig. 5B), stomach, and ileum (Fig. 5C). A 3.1-kb band is detected in spleen (Fig. 5A), prostate, liver (Fig. 5B), duodenum, jejunum, and ileum (Fig. 5C). In skeletal muscle and bladder the calculated size is slightly smaller, 2.9 kb (Fig. 5A). The largest bands are faintly seen in liver (4.8 kb) and heart (4.1 kb) (Fig. 5B).

**Other extrarenal tissues:** **Western blot analysis.** The COOH-terminal UT-A antibody detects multiple bands in each tissue, albeit faintly in spleen, lung, bladder, and gastrointestinal tissues (Fig. 5, D–F). The pattern of bands, as well as their sizes, is different in every tissue, except among some gastrointestinal tissues. A number of tissues have prominent bands between 36 and 64 kDa, including ovary, skeletal muscle (Fig. 5D), prostate, liver (20), and heart (7) (Fig. 5E). Bands between 52 and 57 kDa are common to many tissues including ovary, spleen, lung, skeletal muscle (Fig. 5D), prostate, liver (20), and heart (7) (Fig. 5E), although their intensities are quite variable. The largest band that was commonly detected was around 90 kDa.

Compared to other extrarenal tissues, the intensity of bands in the gastrointestinal tissues is lower (Fig. 5E). A similar pattern including 51- and 76-kDa bands is seen in all except duodenum that instead has 34- and 43-kDa bands.

**UT-B** in Reticulocytes

Total RNA was isolated from rat reticulocytes to show that the RNA bands detected in many organs were not due to retained reticulocytes within the tissues (trapping). The ethidium bromide-stained gel has prominent 18S and 28S ribosomal RNAs and verifies the presence and integrity of the
reticulocyte total RNA (Fig. 6, lane 1). When blotted and probed with a UT-B probe, rat reticulocyte RNA has very faint bands at 2.7 and 3.1 kb as well as rRNA staining (Fig. 6, lane 2). In comparison, reticulocytes isolated from rabbit or human bone marrow show 1.7- (faintly), 2.7-, 3.1- and 4.2-kb bands as well as prominent 18S and 28S rRNA (Fig. 6, lanes 3 and 4).

**UT-B in Kidney**

*Northern blot analysis.* In all kidney subregions, albeit faintly in cortex, the UT-B Probe detects 3.8- and 1.5-kb bands (Fig. 7A).

*Western blot analysis.* Using the UT-B antibody, a group of bands ~50 kDa is prominent in IM mid and IM base and less so in OM (Fig. 7B)(44). A 84-kDa band is best seen in IM base, OM, and cortex. The 98-kDa band detected with this antibody was later shown to be nonspecific as it was seen in the UT-B knockout mouse (52).

**UT-B in Extrarenal Tissues**

*Northern blot analysis.* Panels of total or poly(A) RNAs probed with a UT-B probe show a 3.8-kb band in brain, testis (Fig. 8A), ovary, spleen, lung, bladder, prostate, and liver (Fig. 8B). In lung, liver, and heart, a 2.7-kb band is detected (Fig. 8B). In tissues, such as skeletal muscle (Fig. 8B) and the
gastrointestinal tract (Fig. 8C), the 2.7- and/or 3.8-kb bands are only faintly seen. A 1.5-kb band is seen in some tissues after prolonged exposure (Fig. 8, A–C, inset below corresponding lanes).

**Western blot analysis.** The UT-B antibody detects a similar pattern of bands in cerebellum and cerebrum including two prominent bands at about 47 kDa and others at 39, 50, 55, and 80 kDa (Fig. 8D). Testis has a distinctive 50-kDa band and lighter bands of 36 kDa (doublet) and 104 kDa. In other tissues there are numerous bands, and the pattern is tissue specific (Fig. 8E) (44). The most prominent band(s) were around 50 kDa, and these were most intense in skeletal muscle (43 kDa), prostate (46 kDa), liver (47 and 53 kDa), and heart (46 kDa) (44). A lighter and diffuse group of bands in a similar size range is seen in spleen, lung (44), and bladder. Most tissues also have one or more bands at ~36, 80, or 90 kDa. Bands are detected in all gastrointestinal tissues (except duodenum) of which a 46-kDa band is common (Fig. 8F). Other bands included a 65-kDa band in stomach, jejunum, and colon, a 36-kDa band in stomach, and a ~85-kDa band in stomach (87 kDa) and cecum (83 kDa).

**Immunocytochemistry: Extrarenal Tissues**

**UT-A.** To localize the sites of UT-A expression, immunocytochemical studies were performed on rat tissue sections (Fig. 9) using a polyclonal COOH-terminal UT-A antibody. A transverse section of the gastric glands shows cytoplasmic and nuclear staining in all epithelial cells (Fig. 9A, higher-power exam in a longitudinal section shows that nuclear staining is more prominent in specific cells (Fig. 9B). Villous epithelial cells of the duodenum and jejunum have cytoplasmic staining (Fig. 9, C and D) with a more intense linear pattern just beneath the surface of the villi (Fig. 9, C and D, arrow-
heads) and along the basal membrane (Fig. 9, C and D, arrows). There is diffuse and less intense staining of the lamina propria that is better seen in the duodenum compared with the jejunum. The ileum shows a similar mucosal staining pattern that is more intense at the base of the crypts, although this may be due to higher cell density (Fig. 9E). In ileum, the linear basal epithelial staining is not seen although the cytoplasm and nuclei of the lamina propria are more prominent. The cecum also has the most intense staining in the base of the crypts and a similar linear pattern is detected within the epithelial cell villi (Fig. 9F, arrows). In colon, there is staining of the cytoplasm of Goblet cells and of columnar cells in a linear pattern just beneath the absorptive surface (Fig. 9G, arrows). In the liver, there is heterogeneous staining within hepatocytes with darker staining of the nuclei (Fig. 9H). In the prostate gland, staining is homogeneous in the cytoplasm and very concentrated in the nuclei (Fig. 9I). The urinary bladder shows staining throughout the cytoplasm of the transitional epithelium, and some of the cells are clearly outlined (Fig. 9J). The smooth muscle cells have cytoplasmic and more intense nuclear staining (Fig. 9J, inset), and the single cell layer of the serosal surface is stained (Fig. 9J, inset, arrows). In the left ventricle of heart, there is staining of some large nuclei of myocytes but not in the cytoplasm or endocardium (Fig. 9K). Findings were similar in the right ventricular muscle (not shown). There is staining of lung alveoli in some large cells that are probably Type II pneumocytes (Fig. 9L, arrowheads). The nuclei of these cells are more prominent than the cytoplasm. UT-A can be seen throughout the spleen, concentrated mostly in the nuclei (Fig. 9M). The more prominent staining of lymphoid aggregates (Fig. 9M, asterisk) may be due to cell density. The outermost single cell layer of the capsule is stained (Fig. 9M, arrowheads). In seminiferous tubules (SMTs) of testis, there is cytoplasmic staining of spermatogonic cells (Fig. 9N). It is more intense beginning just beyond the outermost cell layer and lessens toward the center of the lumen. The Leydig cells are negative. There is very prominent staining of UT-A in the Purkinje cells of the cerebellum (Fig. 9O). There is faint

Fig. 9. Immunohistochemistry of UT-A in extrarenal tissues. A–P: immunostaining using the polyclonal antibody to the COOH terminus of UT-A1. Shown are stomach, glandular (transverse) (A); stomach (magnification, ×100) (B); duodenum (C); jejunum (D); ileum (E); cecum (F); colon (G); liver (H); prostate (I); urinary bladder (J); heart, left ventricle (K); lung (L); spleen (M); testis (N); brain, cerebellum (O); and skeletal muscle (P). Magnification is ×20 except in B, which is ×100. Antibody dilution is 1:5,000. See text for description of arrows and arrowheads.
cytoplasmic and scattered nuclear staining in other neurons. There was no significant staining in the cerebral cortex or meninges (not shown). The nuclei of smooth muscle cells stain prominently. UT-A is absent in the cytoplasm (Fig. 9P). There was no staining observed when stomach, duodenum, urinary bladder, heart, brain, or skeletal muscle was probed with preadsorbed UT-A antibody (Fig. 10).

**UT-B.** To localize the sites of UT-B expression, immunocytochemical studies were performed on rat tissue sections (Fig. 11) using a polyclonal COOH-terminal UT-B antibody. No staining is detected in the squamous epithelium of the stomach near the esophagus (Fig. 11A), although there is dark cytoplasmic staining of the gastric epithelial cells (Fig. 11B). The duodenal epithelium shows UT-B throughout, and some areas show a linear pattern just beneath the villous surface (Fig. 11C, arrowheads) and in the basolateral regions (Fig. 11C, arrows). In the jejunum, the cytoplasmic and basal staining of UT-B is less intense, although lateral staining (outlining individual cells) can be clearly seen in several areas (Fig. 11D, arrows). In ileum, the overall staining is greatly diminished except for the cells at the apexes of the villi (Fig. 11E). Cecum has a similar pattern (Fig. 11F). The colon has staining in a basolateral pattern and throughout the cytoplasm of the columnar and Goblet cells, especially those closest to the lumen (Fig. 11G).

In the liver, UT-B appears to be concentrated on the sinusoid lining cells, but not the hepatocytes (Fig. 11H). The prostate gland has staining of UT-B that is most intense on the apical membrane of epithelial cells, less intense in the cytoplasm, and absent in the nuclei (Fig. 11I). The bladder shows homogeneous staining throughout the transitional epithelium, and in some areas, the cells are clearly outlined (Fig. 11J). The serosal surface of the bladder is stained (Fig. 11J, inset, arrows) as well as the smooth muscle cell cytoplasm and nuclei (Fig. 11J, inset). The left ventricle of the heart shows no staining in the cytoplasm of myocytes (Fig. 11K). The focal staining in areas surrounding the myocytes appears to be capillary endothelium (Fig. 11K, arrows). Similar findings were seen in the right ventricle of heart (not shown). The lung has staining of large cells that are probably type II pneumocytes that are most prominent at the cell surfaces and mostly absent in the nuclei (Fig. 11L). The spleen shows patchy staining throughout the red pulp (Fig. 11M). High-power examination reveals that the staining is not simply due to retained red blood cells (not shown). The outermost visceral epithelial cell layer of the capsule shows cytoplasmic staining (Fig. 11M, arrows). In testis, there is faint and diffuse staining of the cytoplasm of some of the outermost layers of cells in the seminiferous tubule and the outlines of some cells are defined (Fig. 11N). There is patchy cytoplasmic staining that continues toward the center of the lumen, although it is not possible to distinguish in what cell type. There was no staining in the interstitial spaces between the tubules (not shown). The brain shows diffuse and faint staining in a reticular pattern throughout the cortex (Fig. 11O). There is staining of the pia mater (Fig. 11O, arrows) but not arachnoid mater (Fig. 11O, arrowheads). In skeletal muscle, there is some staining in the cytoplasm that appears artifactual and none in the nuclei (Fig. 11P). Evidence of UT-B appears to be confined to the vascular structures (Fig. 11P, arrows). There was no staining observed when brain, duodenum, and heart were probed with preadsorbed UT-B antibody (Fig. 12).

**DISCUSSION**

In this study, we detected UT-A and UT-B mRNA and protein forms in whole rat organs and further determined their cellular localization in extrarenal tissues using immunohistochemistry. We found that both UT-A and UT-B exist as
multiple RNA and protein species, having a complex pattern of expression that is unique to each tissue.

**UT-A in Kidney**

We found protein and/or mRNA bands consistent with UT-A1 (4, 34, 42) and UT-A3 (43) in the IM and with UT-A2 (37, 48) in the OM. We also detected a 3.1-kb band in the OM that does not correspond to a known isoform, suggesting that this transcript is either noncoding or the present repertoire of antibodies cannot detect it. We did not find protein bands that correspond to the 2.7-, 3.2-, or 3.6-kb mRNAs detected by UT-A Probe 2 in the IM. Although close in size, the 2.7-kb mRNA is probably not UT-A2b (2.5 kb) (2), because it is not seen in OM (where it is expected). Although we cannot exclude the possibility that any of these mRNAs represent UT-A4, they are all larger than predicted (2.5 kb), and we would expect to detect UT-A4 protein with our antibody.

In cortex, we detected mRNAs and proteins of uncertain identity. Both the 55-kDa protein and 3.1-kb mRNA in cortex are consistent with UT-A2; however, previous studies have not localized it there. Interestingly, a similar result was reported in mouse (13). Others have reported uncharactrized protein bands in cortex of ~60 and ~70 kDa by using a UT-A3-specific antibody and of ~45 and ~50 kDa by using an NH2-terminal UT-A1 antibody (43); however, we did not detect mRNA in that region with our UT-A3 probe. Taken together, these data suggest unique and unidentified UT-A isoforms in cortex (and medulla) that will require further studies to characterize.

**UT-A in Extrarenal Tissues**

We detected UT-A mRNA and protein bands in all tissues examined. We did not find UT-A1 in any extrarenal tissue. Brain was the only tissue that had mRNAs of comparable size to UT-A1; however, the protein bands did not match UT-A1. This suggests the brain may express UT-A1-like proteins, but not UT-A1 itself. We also detected two smaller mRNAs, but their sizes did not match UT-A3. It is possible that brain
expresses UT-A3, but the transcript has a different size due to untranslated regions or that the mRNA represents a UT-A3-like form.

Testis expressed an mRNA band consistent with UT-A5 (9) and a 2.3-kb band that is larger than the expected size of UT-A3 (2.1 kb). Studies by others also have failed to detect the expected 2.1-kb mRNA in testis, yet UT-A3 protein has been immunolocalized there (9). It is possible that the 2.3-kb mRNA could be UT-A3, and for some reason the transcript size is slightly larger than in kidney. We could not use our antibody to support or refute this idea because it does not detect UT-A3 (or UT-A5). We also detect a 2.9-kb mRNA that is similar in size to UT-A2 (3.1 kb) and the UT-A antibody detects several bands of ~55 kDa. These data are consistent with the expression of UT-A2 in testis. Other studies (46) show mRNAs and proteins of different sizes than we found; however, in both cases (Ref. 46 and present study) the proteins are generally larger than would be expected for the mRNA sizes, suggesting that the proteins may be posttranslationally modified.

Our mRNA and protein data together suggest that the majority of the extrarenal forms of UT-A are related to UT-A2 (or UT-A4). Liver and heart also have large mRNA transcripts, but there were no protein bands that matched kidney UT-A1 (20). Furthermore, these mRNAs were detected by only one probe, leading us to conclude that they are UT-A2-like transcripts with longer untranslated regions. We detect a combination of four mRNAs in other extrarenal tissues with a probe that detects UT-A1/A2/A4, indicating that these transcripts may be related to these isoforms. We do not think that the 1.5-kb transcript is UT-A5 because UT-A Probe 2 should not detect it, and the band in testis appears as a diffuse smear, whereas it was sharper in other organs. Also, we did not see the reported 46- and 70-kDa testis proteins (9) in these tissues. In general, we were not able to match the mRNA transcripts with a specific pattern of proteins in any tissues.

Several tissues, including prostate, skeletal muscle, heart (7), and liver (20), had a prominent protein band of ~57 kDa, which is consistent with the size of UT-A2. However, all these tissues had other prominent bands whose sizes were between 36 and 57 kDa, but each was different from one another. This is consistent with either multiple proteins being expressed or tissue-specific expression. Throughout the gut (except duode-
num) UT-A protein appeared homogeneous, but it was difficult to detect in all regions, implying that it has low abundance (20). The pattern of mRNAs, however, was different in each region of the gut. This suggests several mRNAs giving rise to a single protein that has minimal posttranslational modifications. The exception was duodenum that has a single mRNA, which is similar in size to jejumun, ileum, and colon, yet it had a much different pattern of smaller protein forms.

**UT-B in Kidney**

In all subregions (albeit faint in cortex) we detected two mRNAs, including a novel 1.5-kb transcript that either represents one of the cloned rat cDNAs [1,362 bp (24) or 1,412 bp (33, 44, 45, 51)] or a novel, shorter isoform. The larger transcript may arise, as it does in human, from a long 3' UTR (46). We detected a group of protein bands (42–59 kDa) with the greatest intensity and number in IM mid and IM base, which is consistent with the known protein distribution (44) and localization to the descending vasa recta (24).

**UT-B in Extrarenal Organs**

We detected three different rat UT-B mRNAs including novel 1.5- and 2.7-kb transcripts and a 3.8-kb transcript that has been previously reported (46). Possibly, these mRNAs may differ only in UTRs and encode the same protein, as in the case of human UT-B (24), or they may encode different proteins. We have previously reported UT-B protein in rat brain, heart, liver, lung, testis, and colon (44), and this study adds spleen, skeletal muscle, bladder, prostate, and other gut regions. Testis was the only tissue examined that had both a single mRNA species and a single protein band. Using a rat COOH-terminal UT-B antibody designed to a somewhat different peptide sequence; others have found a similarly-sized single band in testis but a smaller, single, glycosylated band in brain and no bands in liver (23).

**Immunocytochemistry**

We found UT-A and UT-B localized to epithelial cells in several organs and throughout the gastrointestinal tract. Although most of the staining was cytoplasmic, we clearly saw sections of apical or basolateral expression, which supports a role for urea transport. Our results are in general agreement with findings in mouse colon (40), except we saw staining of the goblet cells (despite the cytoplasm being obscured by mucus content) but did not see a concentration of stain in the lower regions of the crypts. We did, however, see this pattern for UT-A in ileum and cecum, but for UT-B it was the opposite, with heavier staining toward the lumen.

Other organs showed cytoplasmic epithelial cell staining for both UT-A and UT-B, including bladder transitional epithelium and prostate gland. The finding of cytoplasmic UT-B staining agrees with other studies in rat (39) and mouse (12), although UT-A has not been demonstrated in bladder before, nor has any urea transporter in prostate.

In skeletal and cardiac muscle, we found UT-A in nuclei of myocytes, whereas UT-B expression was confined to vascular structures. We previously showed that UT-A is upregulated in uremic and hypertensive heart, and suggested it may be related to increased polyamine synthesis (7). Finding UT-A within the nuclei argues against this hypothesis, although upregulation could possibly occur in other sites within the cell where basal levels are undetectable.

Of the extrarenal organs examined in this study, liver is the only one with an established physiologic role in urea metabolism. We previously showed UT-A protein is upregulated by uremia and now have localized UT-A to the cytoplasm and nuclei of hepatocytes. UT-B in liver appeared to be confined to sinusoidal cells, which is consistent with a urea transport function.

In lung, UT-A and UT-B are both detected in type II pneumocytes or surfactant secreting cells. Urea can cross the human alveolar membrane (50). The role of urea in surfactant secretion is unknown. The present findings do not support a role for the lung in urea disposal, because we do not observe a uniform staining of Type I cells along the cell-air interface.

In brain, the intense staining for UT-A in the Purkinje cells of the cerebellum suggests cell-specific functions. Until we have a better understanding of urea metabolism in brain, we cannot speculate about the role of UT-A in these cells. We did find UT-B expression in pia where a transport role may be hypothesized. Our present study was less detailed than a previous one that localized UT-B mRNA in neurons and various other cell types throughout the brain, but our immunohistochemical findings are consistent with our Northern and Western blot analysis findings in cerebrum and cerebellum.

In testis, we localized UT-A to several cell layers of the periphery of the SMTs that includes Sertoli cells and developing spermatids. Our present findings differed from a previous study (9) that localized UT-A to residual bodies near the lumen, although neither study detected any significant staining in the interstitium. Our antibody does not detect UT-A5, whose mRNA is localized to the peritubular myoid cells (12) or UT-A3, which is immunolocalized to Sertoli and Leydig cells (9, 46). We also detected UT-B in cells lining the periphery of the tubules (the location of Sertoli cells); however, we detected staining in cytoplasm of the adjacent several cell layers that appear to be developing spermatids. The staining diminished toward the center of the lumen, which suggests these cells no longer express UT-B as they mature. The reason for the differences in the two studies is not clear; however, the antibodies were raised to different COOH-terminal amino acid sequences [19 AA of human UT-B1 (44) vs. 20 AA of rat UT-B (9)]. Perhaps different, and yet unidentified, UT-B proteins are expressed or there may be species-specific differences. Further studies are needed to confirm these findings.

In two organs, spleen and bladder, there was staining for both UT-A and UT-B on the single cell layer of the serosal surface, consistent with a urea transport role where urea moves across the serosal membrane into the fluid of the abdominal cavity and vice versa. It is possible that the other serosal surfaces express urea transporters but we did not identify them because the single-cell-thick layer was damaged during organ harvesting. Demonstrating urea transporters on the parietal epithelium would add further evidence to this role, although this awaits a future study.

In some tissues, the antibody staining was not on the cell membranes, but rather in the cytosol or nucleus. Cytoplasmic localization of urea transporters does not necessarily eliminate a membrane transport role in these organs. Ultrastructural localization studies of UT-A in rat and mouse kidney, where the functional role is established, have shown cytoplasmic
localization (16, 22). The purpose of a transport protein in the nucleus is less obvious. It is possible that UT-A has functions other than urea transport in these tissues or subcellular locations.

There were some limitations to our study. We used a single UT-A antibody that does not detect UT-A3, UT-A5, or UT-A6, although our RNA data (as well as data by others) suggests that these isoforms are limited to only a few tissues. Using additional antibodies against marker proteins in our immunohistochemical studies might confirm subpopulations of cells expressing the urea transporters.

In conclusion, we detected UT-A and/or UT-B mRNA and/or protein forms in a number of organs not typically associated with urea metabolism. UT-A and UT-B were localized to epithelial membranes of some organs, which is consistent with transport function. They were also found in nonepithelial cells or nuclei, which suggests other roles. Further organ-specific physiologic studies are needed to determine these roles, including studies of the recently developed UT-A1/A3, UT-A2, and UT-B null mice. Our data will hopefully support the role of UT-A in epithelial cells or nuclei, which suggests other roles. Further studies of UT-A in the brain and other organs may provide new insights into the function of UT-A.

**REFERENCES**


