Differential Expression of Individual UT-A Urea Transporter Isoforms in Rat Kidney

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Abstract. The rat renal urea transporter UT-A includes four mRNA isoforms: UT-A1, UT-A2, UT-A3, and UT-A4. This study detected by rapid amplification of cDNA ends (RACE), primer extension, and ribonuclease protection assay (RPA) a single transcription start site for UT-A1, UT-A3, and UT-A4, distinct from the one for UT-A2 and identified by 3′-RACE new transcripts of UT-A1, UT-A2, and UT-A3, characterized by alternative 3′ untranslated sequences (UTR). Expression of an alternative 3′UTR resulted in UT-A1 and UT-A2 transcripts that are approximately 400 bp shorter than the original cDNA. These mRNA isoforms (UT-A1b and UT-A2b) were present in low abundance in the inner medulla. Expression of an alternative 3′UTR for UT-A3 resulted in a 3.5 kb transcript (UT-A3b), which is 1.5 kb longer than the original UT-A3 cDNA. UT-A3b mRNA was easily detected by Northern hybridization in the inner medulla. This study examined whether different states of hydration induce homogeneous changes in mRNA expression of individual UT-A isoforms in the kidney. Analysis of UT-A1, UT-A1b, UT-A2, UT-A2b, UT-A3, and UT-A3b mRNA expression in rat kidney revealed that water deprivation markedly increases the relative abundance of UT-A2, UT-A2b, UT-A3, and UT-A3b mRNA in renal inner medulla, whereas UT-A1 and UT-A1b remain almost unchanged. The conclusion is that differential expression of individual UT-A mRNA isoforms occurs in the kidney and probably involves multiple regulatory mechanisms.

Urea plays an important role in the production of concentrated urine and conservation of body water in the kidney. Although urea can diffuse slowly through biologic membranes, rapid movement of urea across tubular and vascular compartments occurs through facilitated transporters. Carrier-mediated urea transport contributes to the generation of the hyperosmolar medullary interstitium, which is necessary for concentrating urea. Two groups of urea transporters have been identified so far in mammals: the vasopressin-regulated renal urea transporter UT-A, and the erythrocyte urea transporter UT-B. Four UT-A isoforms have been cloned and characterized: UT-A1 (1), UT-A2 (2), UT-A3 (3), and UT-A4 (3). All of the UT-A transporters are expressed in the renal medulla. UT-A1 is expressed in the inner medullary collecting duct and mediates the high transepithelial urea transport rate of this tubular segment, and UT-A2 is expressed in the thin descending limb of Henle’s loop (4). The precise segmental distribution of UT-A3 and UT-A4 has not been elucidated. A renal form of the erythrocyte urea transporter UT-B has been identified (5), which is expressed in the vasa recta of the renal medulla (6–8).

Renal expression of UT-A1 and UT-A2 mRNA in vivo has been examined in various conditions. UT-A2 mRNA abundance increases in the outer medulla and the base of the inner medulla after vasopressin administration (9), thirsting (9,10), and low-protein diet for 1 wk (11), without consistent changes in UT-A1 mRNA. These observations suggest that the expression of UT-A1 and UT-A2 in the kidney may be regulated independently. To define how individual UT-A isoforms are regulated in vivo, we extended the analysis of mRNA expression to UT-A3 and UT-A4 in the kidney of rats under different hydration states.

The mechanisms that regulate the long-term expression of these transporters are still unknown, could include transcriptional and posttranscriptional processes, and require a better characterization of the molecular structure of the UT-A transporter. For this purpose, we searched for and identified the transcription start sites of UT-A1, UT-A2, UT-A3, and UT-A4. In addition, we identified new UT-A isoforms that are characterized by alternative 3′ untranslated regions (UTR) UT-A1b, UT-A2b, and UT-A3b, and we tested whether their expression may be susceptible to regulation.

Materials and Methods

Determination of 5′ End UT-A cDNA Sequences

The 5′ end of the UT-A1, UT-A3, and UT-A4 cDNA was determined by 5′ rapid amplification of cDNA ends (RACE), with the reagents provided in the 5′/3′ RACE Kit (Boehringer-Mannheim, Indianapolis, IN), as described previously (3). For 5′-RACE, first-strand cDNA synthesis was performed using an antisense primer spanning nucleotides 610 to 631 of UT-A1 cDNA sequence (Gen-Bank accession number U77971), followed by PCR amplifications using antisense primers spanning nucleotides 481 to 457 or nucleotides 291 to 313 of UT-A1 cDNA. The product of the second PCR amplification was gel purified and sequenced. A similar protocol was used to perform 5′-RACE to search for alternative 5′ ends of UT-A2,
using antisense primers spanning nucleotides 938 to 959, 859 to 879, and 187 to 209 of the UT-A2 cDNA sequence (GenBank accession number U09957).

**Primer Extension Analysis**

Primer extension analysis was performed on 30 μg RNA from rat renal medulla. Oligonucleotide primers 5’-CATCCTCTGAACCTTCGGACGCA-3’ (UT-A1 cDNA, nucleotides 144 to 165) and 5’-CTCTCCAACCTATATTCCAC-3’ (UT-A2 cDNA, nucleotides 188 to 209) were labeled at the 5’ end, using [γ-32P]ATP and T4 polynucleotide kinase. Extension was performed using SuperScript II™ transcriptase (Life Technologies, Gaithersburg, MD) at 55°C. The extension products were separated by electrophoresis on 6% polyacrylamide/7 M urea gels and analyzed by autoradiography.

**RNase Protection Assay**

Ribonuclease protection assay was performed from 30 μg of total RNA from rat inner medulla for UT-A1, UT-A3, and UT-A4, from rat outer medulla for UT-A2, and from yeast tRNA for negative control, using the RPA II kit (Ambion, Austin, TX) and following the instructions of the manufacturer. Sequences that corresponded to the segment −53 to +165 of the beginning of UT-A1 cDNA and its 5’ flanking region, and −22 to +209 of UT-A2 cDNA and its 5’ flanking region were used to make radiolabeled probes. MspI digests of plasmid PBR 322 DNA (New England Biolabs, Beverly, MA) and sequence of plasmid pGMT DNA (Promega, Madison, WI) were used as size markers.

**Identification of Alternative 3’-UTR cDNA Sequences**

The alternative 3’ end sequence of UT-A3 cDNA was identified by 3’-RACE, with the reagents provided in the 5’/3’ RACE Kit, as described previously (3). 3’-RACE was performed with a sense primer corresponding to nucleotides 1764 to 1784 of UT-A3 cDNA (GenBank accession number AF041788). For identification of UT-A1 and UT-A2 alternative 3’-UTR sequence by 3’-RACE, a sense primer corresponding to nucleotides 1418 to 1438 of UT-A2 cDNA, and a nested sense primer corresponding to nucleotides 1573 to 1628 of UT-A2 cDNA were used. The 3’-RACE products were gel purified, subcloned into the pGM plasmid vector (Promega), and sequenced (ABI PRISM Dye Terminator Cycle Sequencing Kit, Perkin Elmer, Foster City, CA). Reverse transcription-PCR (RT-PCR) was performed from 1 μg of total RNA using the TITAN™ RT-PCR System (Boehringer-Mannheim). Analysis of DNA sequences was performed using the Wisconsin Sequence Analysis Package and Lasergene software (DNASTAR Inc., Madison, WI).

**Animal Treatment and Tissue Collection**

Male Sprague-Dawley rats that weighed approximately 250 g were used in these studies. Rats in the control group received water ad libitum, water-deprived rats received 10 ml water per day for 2 d, and water-loaded rats were allowed unlimited access to water with 10% glucose added for 2 d. All animals had unrestricted access to standard rat chow (Prolab Animal Diet RM 3000; PMI Feeds, St Louis, MO). Urine osmolality was measured with a Wescor 5100 C vapor pressure osmometer (Wescor, Logan, UT). Individual rats from each group were randomly assigned to trios of one control, one water-deprived, and one water-loaded animal. Rat kidneys were dissected to separate cortex, outer medulla, and inner medulla, which were rapidly frozen in liquid N2 and stored at −80°C (11). Total RNA from rat kidney was isolated with TRI-REAGENT™ (Boehringer-Mannheim).

**Statistical Analysis**

Changes in mRNA abundance were compared between water-deprived and control rats and between water-loaded and control rats; differences from controls were analyzed by two-tailed, nonpaired t test, with \( P < 0.05 \) indicative of statistical significance.

**Results**

**Identification of Transcription Start Site for UT-A1, UT-A2, UT-A3, and UT-A4**

We performed 5’-RACE, primer extension, and ribonuclease protection analysis to identify the transcription start site of UT-A1, UT-A3, and UT-A4, which have identical 5’-UTR sequences (3). By 5’-RACE, we identified 21 additional nucleotides at the 5’ end of the known rat UT-A1 cDNA sequence (1). The results of primer extension are consistent with a transcription start site at −25, 25 nucleotides upstream of the known beginning of UT-A1, UT-A3, and UT-A4 cDNA (Figure 1A). Ribonuclease protection assay showed more than one protected band, consistent with a site at −25, and other potential transcription start sites contained in the segment −25/+1 (Figure 1B). On the basis of these results, we believe that the transcription of UT-A1, UT-A3, and UT-A4 begins in this region.

Primer extension analysis and ribonuclease protection assay were also performed to find the transcription start site of UT-A2, which we placed within 22 nucleotides upstream of the known beginning of rat UT-A2 cDNA sequence (10) (Figure 2). This sequence is entirely different from the 25 bp segment identified upstream of the known beginning of UT-A1, UT-A3, and UT-A4 cDNA. All together, these results support the

**Northern Hybridization**

RNA samples from cortex, outer medulla, and inner medulla from individual rats in each group were analyzed by Northern hybridization, using 15 μg of total RNA per sample. After separation in 1.0% agarose/2.2 M formaldehyde gels, RNA was immobilized onto a nylon membrane (Hybond-N™, Amersham, Arlington Heights, IL), hybridized with 32P-labeled probes under high stringency conditions (5× SSC, 0.5% sodium dodecyl sulfate [SDS], at 60°C overnight) and washed twice in 2× SSC, 0.1% SDS at room temperature, then once in 0.1× SSC, 0.1% SDS at 60°C. All blots were probed with a rat glyceraldehyde-3-phosphate dehydrogenase cDNA to ensure uniformity of gel loading (12). The full UT-A1 probe included nucleotides 493 to 3262 of UT-A1 cDNA; UT-A3b probe was obtained by PCR amplification of the alternative 3’-UTR sequence of UT-A3 using sense primer 5’-TGACCAAGCTGGAGGCTCCTA-3’ and antisense primer 5’-GGGCACACAGCTCAAACCTTTAGAAAC-3’ (855 bp); UT-A2b probe was obtained by PCR amplification of the alternative 3’-UTR sequence of UT-A2 using sense primer 5’-TCTCTCACAGGAAAAGGCA-3’ and antisense primer 5’-TCAGGAATCTAAAAGTGCA-3’ (310 bp). The cDNA templates were labeled with 32P-dCTP using the random primer technique (DNA Random Primed Labeling Kit, Boehringer-Mannheim). Autoradiograms of the hybridized membranes were examined by densitometry to quantify the intensity of the signals and to determine the relative abundance of mRNA in different states of hydration. To control for loading, densitometric measurements were normalized for the glyceraldehyde-3-phosphate dehydrogenase signal in each lane.
hypothesis that the transcription start site of UT-A1, UT-A3, and UT-A4 is distinct from the transcription start site of UT-A2.

Identification of Alternative 3′-UTR Sequences for UT-A1, UT-A2, and UT-A3

In a previous study (3) using Northern hybridization with a full-length UT-A1 cDNA probe as well as a probe specific for the 3′-UTR of UT-A3, we detected a 3.6- to 3.7-kb band in the kidney, which did not match the size of any previously identified UT-A isoform. We hypothesized that it may represent a UT-A3 variant and tried to clone it by 3′-RACE, using nested primers from the 3′ end of UT-A3 cDNA. We identified a 3,472-bp cDNA, which turned out to be UT-A3 with a 3′-UTR longer than the previously cloned UT-A3 cDNA (3). The sequence of the extended 3′-UTR is shown in Figure 3A, and we refer to this UT-A3 variant as UT-A3b.

We hypothesized that there may be alternative 3′-UTR sequences expressed in other UT-A isoforms and tried to identify them. We performed 3′-RACE with primers from the cDNA sequence corresponding to the C-terminal portion of UT-A2, and we identified a 403-bp novel sequence. By RT-PCR using sense primers specific for either UT-A1 or UT-A2 and sequencing of the RT-PCR products, we determined that this sequence was expressed as an alternative 3′-UTR in UT-A1 and UT-A2 (Figure 3B). This 3′-UTR is shorter than the one described previously (approximately 800 bp) (10), to which it does not show any homology. The UT-A1 transcript including the alternative 3′-UTR (UT-A1b) shows that splicing of this sequence occurs at nucleotide 3189 of the original sequence. In the UT-A2 transcript, splicing occurs at nucleotide 2144 of the original sequence. Figure 4 shows the different UT-A cDNA isoforms aligned to highlight regions of identity, predicted transcription start sites, and alternative 3′-UTR.
Expression of UT-A Transporters in Rat Kidney in Different Hydration States

By Northern hybridization using specific probes, UT-A3b is expressed in substantial amounts in the inner medulla as a 3.6- to 3.7-kb band (Figure 5B); UT-A1b and UT-A2b appear as bands of 3.5 and 2.5 kb, respectively (Figure 5C). UT-A1b expression was difficult to detect but seems to be restricted to the inner medulla. UT-A2b was weak but generally detectable in the outer medulla and inner medulla of most rats studied.

Although previous studies demonstrated increased expression of UT-A2 in the renal medulla of water-deprived rats, such analysis preceded the identification of UT-A3, UT-A4, UT-A3b, UT-A1b, and UT-A2b. We sought evidence for regulated mRNA expression of these recently identified UT-A isoforms in the kidney of rats in different states of hydration.

Water-loaded rats produced 46 ± 7 ml/d of urine compared with 12 ± 2 ml/d by the control rats (n = 4, P = 0.01); the water-deprived rats produced less than 1 ml urine/d by the second day of water deprivation. Urine osmolality was 450 ± 100 mOsm/kg H₂O in the water-loaded rats, 1320 ± 130 mOsm/kg H₂O in the control rats, and 1860 ± 105 mOsm/kg H₂O in the water-deprived rats (n = 4, P < 0.01 by ANOVA).

Figure 5 shows representative Northern hybridization, indicative of the expression patterns of UT-A mRNA isoforms in rat kidneys of water-deprived and water-loaded rats compared with control rats. We confirmed previous observations that UT-A4 is expressed in very low abundance in the rat kidney (3). Therefore, we decided to exclude UT-A4 from our analysis and focused on the relative abundance of UT-A1, UT-A1b, UT-A2, UT-A2b, UT-A3, and UT-A3b. In the outer medulla, water deprivation significantly increased expression of UT-A2 (1.99 ± 0.46-fold, P < 0.05) and of UT-A2b mRNA (2.31 ± 0.95-fold, P < 0.05). Water loading significantly decreased the expression of UT-A2 (0.49 ± 0.13-fold, P < 0.05); the decrease in UT-A2b expression was not significant. Figure 6 shows changes of UT-A mRNA isoform expression in the

Figure 2. (A) Primer extension analysis of UT-A1 transcript, showing a product of 225 nucleotides obtained with total RNA from the outer medulla (Rat O.M.). A reaction without template was used as negative control (No RNA). Sequence of pGEMT used as a size marker is shown on the right. (B) Ribonuclease protection assay showing the largest protected band of 225 nucleotides and other smaller protected bands obtained with total RNA from outer medulla (Rat O.M.). A reaction with yeast tRNA was used as a negative control (tRNA). Sequence of pGEMT used as a size marker is shown on the right. (C) The sequence including transcription start sites (arrow) is shown (underlined) at the 5’ end of UT-A1 cDNA.
inner medulla of water-deprived and water-loaded animals, relative to the control group, and illustrates the relative abundance of the various UT-A isoforms. Although the changes were not significant, UT-A1 and UT-A1b were increased by water deprivation; UT-A1 was somewhat decreased by water loading, whereas UT-A1b was not. In the inner medulla, water deprivation significantly increased the expression of UT-A2 (P < 0.01) and of UT-A2b (P < 0.05). Water loading did not significantly affect UT-A2, whereas a small but significant increase was observed for UT-A2b (P < 0.05). UT-A3 mRNA expression was detected only in the inner medulla; it was significantly increased by water deprivation (P < 0.05) and was significantly decreased by water loading (P < 0.05). UT-A3b mRNA abundance was also increased by water deprivation, although the change did not reach statistical significance.

Discussion

In this study, we report the identification of the transcription start site of UT-A1, UT-A2, UT-A3, and UT-A4. We describe new cDNA variants of previously cloned UT-A isoforms, characterized by alternative 3'-UTR sequences: UT-A1b, UT-A2b, and UT-A3b. We examine the distribution of their mRNA expression in the kidney. We also examine the effect of different states of hydration on the renal mRNA expression of UT-A1, UT-A1b, UT-A2, UT-A2b, UT-A3, and UT-A3b.

Although it is not clear what the physiologic significance of multiple UT-A isoforms is, including the new transcripts with alternative 3'-UTR sequence described in this article, the mRNA abundance of most isoforms in the renal medulla is substantial. This seeming multiplicity suggests that their role may be important for renal function and that their regulation may be complex. The short-term regulation of the functional activity of the four UT-A proteins has been characterized only partially, and even less is known about the mechanisms involved in the long-term regulation of mRNA expression of individual UT-A transporters in vivo. The four UT-A isoforms are highly homologous, suggesting that they are encoded by a single gene. We have evidence that the transcription start site of UT-A1, UT-A3, and UT-A4 is different from the transcription start site of UT-A2. Different transcription start sites may

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**Figure 4.** UT-A cDNA isoforms. Regions of identity among isoforms are indicated by different patterns. Orientation of transcription and transcription sites are indicated by arrow lines.
be associated with different transcriptional regulatory mechanisms affecting expression of individual UT-A isoforms.

It has previously been noted that dehydration increases expression of UT-A2 mRNA in the kidney while leaving UT-A1 mRNA expression unchanged (10,13). Our analysis confirms these previous reports and provides new information. Our results show that UT-A2 and UT-A2b expression increases in the outer and inner medulla of water-deprived rats and decreases in the outer medulla of water-loaded rats. Although UT-A1 and UT-A1b also show a tendency to increase with water deprivation, the changes are small and do not reach statistical significance. It is interesting that we find that expression of the UT-A3 and UT-A3b transcripts is increased by water deprivation and UT-A3 is decreased by water loading in the inner medulla. The UT-A3 and UT-A3b cDNA are identical to the 5' half of UT-A1, although their 3' UTR is different. However, they have the same transcription start site as UT-A1. It is surprising that only the expression of UT-A3 but not of UT-A1 changes significantly in response to water deprivation. This finding suggests that transcriptional as well as posttranscriptional mechanisms may participate in regulating expression of these UT-A isoforms.

As for the nature of the mechanisms that regulate UT-A expression, they may be related to changes in the tonicity of the renal medulla, which are known to influence transcription of other medullary transporter genes (14–16). Whether vasopressin is involved in the long-term regulation of UT-A transporter expression remains to be clarified. It is widely known that 5' and 3' UTR sequences may be involved in regulating gene expression by mRNA stability and translational efficiency (17–20). Although regulation of promoter activity could influence transcription, modulation of pre-mRNA splicing or mRNA stability could provide additional means of adjusting the relative abundance of UT-A isoforms in response to specific stimuli (21–23). It is possible that UTR sequences may play a role in posttranscriptional regulation of UT-A isoform expression. Additional studies are necessary to identify which mechanisms are involved in the long-term regulation of the UT-A urea transporter expression in the kidney.

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