Structure and characterization of the mouse UT-A gene (Slc14a2)

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Fenton, R. A., C. A. Cottingham, G. S. Stewart, A. Howorth, J. A. Hewitt, and C. P. Smith. Structure and characterization of the mouse UT-A gene (Slc14a2). Am J Physiol Renal Physiol 282: F630–F638, 2002.—The movement of urea across plasma membranes is modulated by facilitated urea transporter proteins. These proteins are the products of two closely related genes, termed UT-A (Slc14a2) and UT-B (Slc14a1). By genomic library screening and P1 artificial chromosome “shotgun” sequencing, we have determined the structure of the mouse UT-A gene. The gene is >300 kb in length, contains 24 exons, and has 2 distinct promoters. Flanking the 5′-region of the gene is the UT-Aα promoter that regulates transcription of UT-A1 and UT-A3. The second promoter, termed UT-Aβ, is present in intron 13 and regulates transcription of UT-A2. cAMP agonists (100 μM dibutyl cAMP, 25 μM forskolin, 0.5 mM IBMX) increased the activity of a 2.2-kb UT-Aα promoter construct 6.2-fold (from 0.026 ± 0.003 to 0.160 ± 0.004, relative light units (RLU)/μg protein) and a 2.4-kb UT-AB promoter construct 9.5-fold (from 0.020 ± 0.002 to 0.190 ± 0.043 RLU/μg protein) above that in untreated controls. Interestingly, only the UT-AB promoter contained consensus sequences for CREs and deletion of these elements abolished cAMP sensitivity. Increasing the tonicity of culture medium from 300 to 600 mosmol/kgH2O with NaCl caused a significant increase (from 0.060 ± 0.004 to 0.095 ± 0.010 RLU/μg protein) in UT-Aα promoter activity but had no effect on the UT-Aβ promoter. A toxicity-responsive enhancer was identified in UT-Aα and is suggested to be responsible for mediating this effect. Levels of UT-A2 and UT-A3 mRNA were increased in thirsted mice compared with control animals, indicating that the activities of both promoters are likely to be elevated during prolonged antiuresis.

Facilitative urea transporters belonging to the UT-A family of proteins regulate the movement of urea across biological membranes (24, 28). In rats and humans, these proteins are the products of a single gene (Slc14a2) (1, 18). Seven cDNAs encoding four urea transporters, UT-A1, UT-A2, UT-A3, and UT-A4, have been characterized in rat kidney (2, 28), and a cDNA encoding a novel isoform, UT-A5, has been isolated from mouse testes (8).

UT-A proteins expressed in the kidney play a critical role in generating the hypertonic medulla, which is an integral component of the urinary concentrating mechanism. In rat, thirsting increases levels of UT-A2 and UT-A3 mRNA (2, 27), and parallel changes have been observed in UT-A2 protein (32). The increase in UT-A2 mRNA is mediated in part by arginine vasopressin (AVP), because Brattleboro rats, which lack endogenous AVP, do not show this response. Only when AVP or 1-desamino-8-D-arginine vasopressin is administered is the increase in UT-A2 mRNA then observed (21, 26). These changes are possibly mediated via vasopressin V2 receptors coupled to cAMP (21). A similar mechanism may also be responsible for the increase in UT-A3 mRNA, although this remains to be determined.

We have previously mapped the mouse UT-A gene to chromosome 18 and shown that it is arranged in tandem with the UT-B gene (Slc14a1) (7). To understand how UT-A mRNAs are differentially regulated, in particular during water restriction, and as a prerequisite for groups wishing to perform mouse knockout studies, we determined the structure of the mouse UT-A gene and have conducted studies to characterize its promoters.

METHODS

Isolation of the mouse UT-A gene. cDNAs encoding UT-A1 (GenBank accession no. AF366052) and UT-A2 (GenBank accession no. AF367359) were isolated from a mouse kidney inner medulla (IM) cDNA library and sequenced as previously described (Fenton RA, Stewart GS, Carpenter B, Howorth A, Cooper GJ, and Smith CP, unpublished observations). A XPXII/129sv mouse genomic library (Stratagene) and a P1 artificial chromosome (PAC) genomic library, obtained from the Human Genome Mapping Project (HGMP Resource Centre), were screened using the following 32P-labeled mouse UT-A cDNA probes: probe 1, nucleotides 1–222 of mouse UT-A3 (GenBank accession no. AF258602); probe 2, nucleotides 1–176 of mouse UT-A5 (GenBank accession no. AF258601); probe 3, nucleotides 1,745–1,950 of
mouse UT-A3; probe 4, nucleotides 1–799 of mouse UT-A2; and probe 5, nucleotides 3,378–3,956 of mouse UT-A1. Filters were incubated overnight at 48°C in hybridization solution containing 7% SDS, 0.5 M NaHPO₄ (pH 7.2), 1 mM EDTA, and 50 mM BSA, supplemented with denatured salmon sperm DNA (10 μg/ml hybridization solution). Filter washes were in 0.1× standard sodium citrate, 0.1% SDS at 65°C. Phage clones were further analyzed by restriction digest and Southern blotting. Four lambda clones, MUT1 (16 kb), MUT2 (15.1 kb), MUT4 (4.5 kb), and MUT9 (15.2 kb), were identified on the basis of their binding to the panel of cDNA probes. These were digested with SalI, XbaI, and SacI, respectively, subcloned into pBluescript SK−, and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

PAC clone 459-11 was found to contain the majority of the UT-A gene and was “shotgun” sequenced by Lark Technologies (Saffron Walden, UK). Four plates containing 96 clones were sequenced using pUC forward and reverse primers and Big Dye Terminator Cycle Sequencing kits (PE Applied Biosystems). The assembled 768 reads that were screened for vector sequence and assembled along with the four lambda phage genomic clones into contiguous sequences using the PHRED-PHRAP package (GCG). Exon/intron boundaries were determined by sequence comparison with mouse UT-A cDNAs using the BLAST 2 program [National Center for Biotechnology Information (NCBI)] (30). Exon/intron boundaries that were not identified by this method were determined by “primer walking” off PAC clone DNA using exon-specific primers. Some intronic distances were determined using the NCBI program Trace Archive MEGABLAST search database (http://www.ncbi.nlm.nih.gov/blast/mmtrace.html) and unedited Mus musculus shotgun sequence generated from the mouse genome project. Potential cis-regulatory elements were identified using Promoter Inspector software (Genomatix) (25).

Promoter-reporter plasmid construction. To analyze promoter activity, promoter constructs were engineered into the pGL3 Basic reporter vector (Promega). A 2,239-bp fragment of the UT-Aα promoter (UT-Aα-2.2) was generated from MUT9 by PCR using Pfu polymerase (Stratagene) and the primer pair 5′-GACTCCTATAGGGCCGTG and 5′-Xhol-CAGCCTAGAGAAGAATGGT. A 2,438-bp (UT-Aβ-2.4) fragment of the UT-Aβ promoter was generated from MUT1 by PCR using Pfu polymerase and primer pair 5′-Xhol-CTTCCGTAAGAACAGATAG and 5′-BglII-GTGATATGACCTAACGCGTGT. Primers 5′-Xhol-GTGATATGACCTAACGCGTGT and 5′-XhoI-CTTCCGTAAGAACAGATAG, each in combination with primer 5′-BglII-GTGATATGACCTAACGCGTGT, were used to generate, respectively, the following truncated UT-Aβ promoter constructs: 857 bp (UT-Aβ-0.8), 533 bp (UT-Aβ-0.5), and 285 bp (UT-Aβ-0.3). All products were directionally cloned into pGL3 Basic and sequenced.

cell culture, transfection, and reporter gene assay. Madin-Darby canine kidney (MDCK) cells were grown in 37°C/5% CO₂ in MEM (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 0.5% penicillin/streptomycin (Sigma), 1% l-glutamine (200 mM; GIBCO BRL), and 1% nonessential amino acids (Sigma). Twenty-four hours prior to transfection, cells were seeded in 24-well plates at a density of 8 × 10⁴ cells/ml. Cells (∼60% confluent) were transfected using Transfast reagent (Promega) with equimolar amounts of UT-A plasmid constructs (5 pmol) and, as a control for transfection efficiency, 5 ng of pRLB19 (11). pRLB19 was a kind gift from Dr. Arlyn Garcia Perez, National Heart, Lung, and Blood Institute, National Institutes of Health. Transfections were performed according to the manufacturer’s recommended protocol. MEM was removed 24 h after transfection, the cells were washed twice with prewarmed PBS, and 1 ml of fresh media was added. At this point, in experiments employing hypertonic media, cells were exposed to medium made hypertonic (600 mosmol/kgH₂O) by the addition of NaCl. In experiments employing cAMP, cells were subjected to media supplemented with dibutyryl cAMP (100 μM), forskolin (25 μM), and IBMX (0.5 mM). After a further 24 h, cells were washed with PBS, 100 μl of passive lysis buffer (Promega) was added to each well, and plates were shaken on an orbital shaker for 30 min at room temperature. The resulting cell lysate was centrifuged at 10,000 g for 1 min and analyzed for total protein using a protein assay kit with γ-globulin as a standard (Bio-Rad). Firefly and Renilla luciferase activities, in relative light units (RLU), were determined using the Dual Luciferase Reporter Assay System (Promega) and an EG&G Berthold Microlumat 96P luminometer.

Experimental firefly luciferase activity was normalized to Renilla luciferase activity (calculated as RLU/μg of cell protein). The response of the UT-A constructs to different experimental conditions was determined by comparing the ratio of measurements of firefly luciferase to Renilla luciferase between the experimental groups.

Fluid-restriction protocol. Adult male MF1 mice were used in this study and had unrestricted access to standard 18% protein mouse chow (Special Diet Services). Control mice received water ad libitum, and thirsted mice had no access to water for 20 h. Mice were killed by cervical dislocation, and blood and urine were immediately collected, respectively, by cardiac puncture or by puncturing the urinary bladder. Kidney IMs were removed and snap-frozen in liquid N₂. The osmolality of serum and urine samples was determined using a Roebling microosmosmeter. Serum and urine urea nitrogen concentrations were determined using the Sigma Diagnostics BUN (end point) reagent kit according to the manufacturer’s protocol.

Northern blot analysis. Total RNA was isolated from individual kidney IMs by the guanidine isothiocyanate method followed by ultracentrifugation as previously described (27). Total RNA (8 μg/ lane) was separated in a 1% agarose gel in the presence of 2.2 M formaldehyde and transferred to Hybond-N filters (Amersham Pharmacia Biotech). Filters were probed, using a32P-labeled full-length mouse UT-A1 cDNA. Hybridization was for 16 h at 42°C (50% formamide) and washing at 65°C in 0.1× standard sodium citrate, 0.1% SDS. To control for equal loading of RNA, membranes were subsequently probed with a32P-labeled mouse glyceraldehyde-3-phosphate dehydrogenase cDNA probe. Autoradiographs were analyzed by densitometry (LAS-100 camera using AIDA software, FujiFilm) to quantify the relative abundance of different mRNA transcripts under different hydration states.

Statistical analysis. For experiments involving two groups, the unpaired Student’s t-test was used. For experiments involving three or more groups, one-way ANOVA combined with the Bonferroni post hoc test was used. Groups were deemed statistically significant if P < 0.05.

RESULTS

Structure of the UT-A gene. The UT-A gene consists of 24 exons, ranging in size from 50 (exons 12 and 23) to 965 bp (exon 24), distributed over ~300 kb of DNA (Fig. 1B). All exon/intron boundaries contained a 5′-donor gt and a 3′-acceptor ag (Table 1). The first five exons of the UT-A gene are present in the transcripts UT-A1
and UT-A3; the first three form the 5'-untranslated region (UTR) of these transcripts. The putative translational start site for these transcripts is located in exon 4 (sequence caaATG). Exon 6 forms the unique 5'-UTR of the UT-A5 transcript. This is one of three unique exons not present in the largest transcript, UT-A1. Exon 7 contains the translation start site of UT-A5 (sequence gtgATG).

Exons 8–12 are common to UT-A1, UT-A3, and UT-A5. Exon 13 is of particular interest. The overall size of this exon is based on the first used polyadenylation signal (ATTAAA) of UT-A3 and UT-A5. The translation stop codon for these two transcripts is located at nucleotides 181–183 of the exon (TAG). All of the exon is present in the UT-A3 and UT-A5 transcripts, but in the UT-A1 transcript only the first 175 bp are present, and, thereafter, exon 15 is spliced in (see Fig. 1C).

Exon 14 is the unique 5'-UTR of the UT-A2 transcript. Exon 17 contains the putative translation start site of UT-A2 (sequence accATG). Exons 18–24 are present in UT-A1 and UT-A2. The stop codon for UT-A1 and UT-A2 (TTA) starts at bp 198 of exon 24. Again, the overall size of this exon is based on the first used polyadenylation signal (ATTAAA) of UT-A1 and UT-A2.

**UT-A promoters.** Multiple transcriptional start sites for the UT-A1 and UT-A3 transcripts were identified (Fenton RA, Stewart GS, Carpenter B, Howorth A, Cooper GJ, and Smith CP, unpublished observations). Sequence analysis of the 5' sequence flanking exon 1, termed UT-Aα, (GenBank accession no. AF367361) revealed that it resembled a TATA-less promoter, in that it lacked a TATA-box consensus sequence. However, it did contain three CCAAT boxes, three Sp1 transcription factor binding sites, and a consensus site for a transcriptional initiator protein [Inr; (C/T)(C/T)(A/T)(A/C/T)(C/T)] (Fig. 2A). Also present were consensus sequences for other well-characterized transcription factors (see Fig. 2A), including two glucocorticoid response elements (GRE) and, interestingly, a tonicity-responsive enhancer/osmotic response element (TonE/ORE) (10, 23, 29). Of note is the absence of any consensus sequences for cAMP responsive elements (CRE) or CRE binding proteins (3).

A single transcriptional start site for the UT-A2 transcript was identified by primer extension and 5'
UT-A UREA TRANSPORTER GENE

Table 1. Intron/exon boundaries for the 24 exons of the mouse UT-A gene

<table>
<thead>
<tr>
<th>Exon No.</th>
<th>Amino Acid</th>
<th>Length, bp</th>
<th>Splice Acceptor</th>
<th>Splice Donor</th>
<th>Intron Size, bp</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>5'-UTR</td>
<td>149</td>
<td>-</td>
<td>TGGGAAGtaag</td>
<td>&gt;13,000</td>
</tr>
<tr>
<td>2</td>
<td>5'-UTR</td>
<td>132</td>
<td>ATCTTT</td>
<td>GTGACTgtag</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>5'-UTR</td>
<td>66</td>
<td>tctagATCTTT</td>
<td>GAAACTgtag</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1-159</td>
<td>187</td>
<td>tctagATCACAT</td>
<td>GAAAAGgtag</td>
<td>2,444</td>
</tr>
<tr>
<td>5</td>
<td>60-119</td>
<td>181</td>
<td>atctagGATCCTC</td>
<td>TGAAAGgtag</td>
<td>455</td>
</tr>
<tr>
<td>6</td>
<td>5'-UTR</td>
<td>186</td>
<td>cgtagCTGTG</td>
<td>CACCGgtacta</td>
<td>&gt;500</td>
</tr>
<tr>
<td>7</td>
<td>120-183</td>
<td>190</td>
<td>tgtcagATAAACG</td>
<td>GGACAGgtagt</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>8</td>
<td>184-226</td>
<td>129</td>
<td>acccagGTGAGCC</td>
<td>GGCCTGgtag</td>
<td>3,433</td>
</tr>
<tr>
<td>9</td>
<td>227-290</td>
<td>193</td>
<td>ctacagCCCCCCT</td>
<td>CTCTTtgtag</td>
<td>6,187</td>
</tr>
<tr>
<td>10</td>
<td>291-339</td>
<td>148</td>
<td>tgtcagCTGTG</td>
<td>TACCAGgtagt</td>
<td>1,050</td>
</tr>
<tr>
<td>11</td>
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<td>135</td>
<td>tgtcagCTGTG</td>
<td>TCTCTGgtag</td>
<td>373</td>
</tr>
<tr>
<td>12</td>
<td>385-401</td>
<td>50</td>
<td>ecacagCCCTCT</td>
<td>GCAGTgtag</td>
<td>481</td>
</tr>
<tr>
<td>13‡</td>
<td>402-459</td>
<td>366</td>
<td>tttagacTTG</td>
<td>ATTAA</td>
<td>4,144</td>
</tr>
<tr>
<td>14‡</td>
<td>5'-UTR</td>
<td>683</td>
<td>ggttacGTATA</td>
<td>GCCCGgtaga</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>15</td>
<td>480-601</td>
<td>126</td>
<td>ggttagGTGAGCA</td>
<td>GCAAGgtctg</td>
<td>2,255</td>
</tr>
<tr>
<td>16</td>
<td>502-529</td>
<td>83</td>
<td>tctacagGTCTT</td>
<td>CTCTTtgtag</td>
<td>735</td>
</tr>
<tr>
<td>17</td>
<td>530-582</td>
<td>160</td>
<td>tgtcagGACCTCG</td>
<td>TAAAGgtacta</td>
<td>697</td>
</tr>
<tr>
<td>18</td>
<td>583-646</td>
<td>190</td>
<td>tgtcagATAAAC</td>
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</tr>
<tr>
<td>19</td>
<td>647-689</td>
<td>129</td>
<td>cccacagGTCAA</td>
<td>GACTTgtag</td>
<td>871</td>
</tr>
<tr>
<td>20</td>
<td>690-753</td>
<td>193</td>
<td>cattacCCCCCAT</td>
<td>TCTCTGgtag</td>
<td>1,520</td>
</tr>
<tr>
<td>21</td>
<td>754-802</td>
<td>148</td>
<td>ttacagCTCTCG</td>
<td>TACGAgtctg</td>
<td>1,107</td>
</tr>
<tr>
<td>22</td>
<td>803-847</td>
<td>135</td>
<td>cctacagtCTCG</td>
<td>CCTGgtag</td>
<td>1,679</td>
</tr>
<tr>
<td>23</td>
<td>848-864</td>
<td>50</td>
<td>ttacagCTCTCG</td>
<td>TCTGgtag</td>
<td>2,317</td>
</tr>
<tr>
<td>24‡§</td>
<td>865-930</td>
<td>965</td>
<td>cttcagTTG</td>
<td>ATTAA</td>
<td>-</td>
</tr>
</tbody>
</table>

Intron sequences are shown in lowercase and exon sequences in uppercase. Consensus splice site nucleotides are underlined. Amino acid refers to the amino acid no. of the largest transcript, urea transporter UT-A1. Intron size refers to sizes of known intronic regions; dashes represent unknown distances. †Exon 6 is the unique 5'-untranslated region (UTR) of UT-A5. ‡The size of exon 13 refers to the first used polyadenylation signal of UT-A3 and UT-A5. §Exon 14 is the unique 5'-UTR of UT-A2. ¶The size of exon 24 refers to the first used polyadenylation signal of UT-A1 and UT-A2.

rapid amplification of DNA ends (Fenton RA, Stewart GS, Carpenter B, Howorth A, Cooper GJ, and Smith CP, unpublished observations). The 4,144 bp of sequence upstream of this start site were sequenced. All of this intron (intron 13) was initially thought to represent an internal promoter. Preliminary studies showed that this fragment, when subcloned into the pGL3 Basic vector, had inherent promoter activity (results not shown). However, the recent identification in rats of an alternative 1,500-bp 3' UTR for UT-A3 (UT-A3b) (2) that extends into this intron prompted us to truncate the construct so as to exclude this putative exon. Therefore, of the initial 4,144 bp of sequence, 2,458 bp flanking exon 14 were functionally analyzed. This sequence, termed UT-AB (GenBank accession no. AF367360), contains a consensus TATA-box sequence (TATATA) 27 bp upstream of the UT-A2 transcription start site (+1). Two inserted CCAAT boxes (ATTG) were identified at −206 and at −2,295. Several other consensus sequences for well-characterized transcription factors were identified (Fig. 2B), including five CREs.

Functional analysis of the UT-A promoters. The activities of the UT-Aα and UT-Aβ promoters were measured using MDCK cells transiently transfected with different promoter constructs. Constructs were tested for both basal and stimulated luciferase activity. The 2.2-kb UT-Aα construct (UT-Aα-2.2) induced basal promoter activity [0.026 ± 0.003 (SD) RLU/μg protein, n = 3] 5.2-fold greater than that with pGL3 Basic without an insert (Fig. 3B). The 2.4-kb UT-AB (UT-AB-2.4) construct induced basal promoter activity (0.020 ± 0.002 RLU/μg protein) 4.0-fold greater than that with pGL3 Basic without an insert. Interestingly, the smaller UT-AB constructs induced higher basal promoter activity than did the largest construct (Fig. 3B), suggesting possible negative regulation by suppressor or silencer elements (15).

In the UT-Aβ promoter region there are five consensus sequences for CREs, but in the UT-Aα promoter no consensus CREs are present. A mixture of cAMP agonists increased the activity of the 2.4-kb UT-Aβ construct (0.190 ± 0.043 RLU/μg protein) 9.5-fold more than in untreated controls (Fig. 3B). Interestingly, the activity of the 2.2-kb UT-Aα construct (0.160 ± 0.004 RLU/μg protein), when tested in the same experiment, was stimulated 6.2-fold by the mixture of cAMP agonists (Fig. 3A).

The effect of deleting the CREs on stimulation of UT-Aβ by cAMP was also tested. cAMP had no effect on the luciferase activity of the 0.3-kb UT-Aβ construct that contained no CREs. All other constructs contained CREs, and stimulation by cAMP induced a significant (P < 0.001) increase in luciferase activity (Fig. 3B). Progressively larger constructs, containing more CRE consensus sequences, induced stepwise increases in luciferase activity. Interestingly, there was a large difference in response to cAMP between the 0.8- and the 2.4-kb UT-Aβ constructs, suggesting that CRE 4 and 5 have a relatively large influence on promoter activity compared with the other CREs. Together, these data indicate that the CREs found in the 5'-flanking region of UT-A2, particularly between approximately −800 and −2,400, are responsive to cAMP and
Fig. 2. A: the UT-A1 promoter region. The 5' cDNA sequence of mouse UT-A1 is shaded. CAAT boxes are highlighted in bold, and other potential cis-elements are underlined. AP-1, activator protein 1; GRE, glucocorticoid response elements; TonE, tonicity-responsive enhancer. B: UT-A2 promoter region. The 5' cDNA sequence of mouse UT-A2 is shaded. A TATA-box consensus sequence is highlighted in bold, and other consensuses sites for known transcription factors and potential cis-elements are underlined. CRE, cAMP responsive elements.
Effect of fluid restriction on UT-A mRNA expression.

To determine whether changes in UT-A promoter activity conferred changes to UT-A mRNA levels in vivo, we compared UT-A mRNA levels in kidney IMs from control mice and mice deprived of water for 20 h. Urine osmolalities were significantly higher for fluid-restricted animals (3,480 ± 355 mosmol/kgH2O) compared with control mice (1,973 ± 560 mosmol/kgH2O). Northern blot analysis (Fig. 5) of mouse kidney IM showed that the levels of the UT-A2 (3.1-kb) and UT-A3 (2.1-kb) transcripts increased, compared with non-fluid-restricted controls (P < 0.05) in response to fluid deprivation (summarized in Fig. 6). In contrast, neither UT-A1 mRNA (4.1-kb) nor glyceraldehyde-3-phosphate dehydrogenase mRNA levels were different between experimental groups.

DISCUSSION

By screening a λFIXII/129sv genomic library, shotgun sequencing of a 180-kb PAC clone, and primer walking of PAC clone DNA, we have determined the structure of the mouse UT-A gene. The gene spans ∼300 kb of chromosome 18 (7) and includes 24 exons. Transcription is driven by two promoters, one proximal to exon 1 (termed UT-Aa), driving transcription of UT-A1 and UT-A3, and an internal promoter (termed UT-Ab), flanked by exons 14 and 15, driving transcription of UT-A2.

UT-A1, the largest transcript derived from the UT-A gene, comprises exons 1–5, 7–12, part of exon 13, and exons 15–24. Exon 13 is intriguing because it is a split exon. The first 175 bp are present in the mature UT-A1 transcript as a coding sequence, whereas the complete exon is present in UT-A3 and UT-A5 and codes for the terminal amino acids and the 3'-UTR containing the polyadenylation consensus. Given its dual role, this exon is likely to influence the transcription of UT-A1 because the efficiency of an RNA splicing event is determined by a number of elements often present within an exon or the flanking intron (16). These...

Fig. 3. The effect of cAMP on the UT-A promoters. Promoter activity induced by exposure of cells for 24 h to a mixture of cAMP agonists is shown as a ratio between firefly and Renilla luciferase activity [relative light units (RLU)/μg protein]. Values are means ± SD; n = 3. A: promoter activity in Madin-Darby canine kidney (MDCK) cells transfected with the UT-Aa construct. B: promoter activity in MDCK cells transfected with different UT-Ab constructs. ***Significant increase in promoter activity compared with control. **Significant increase in effects of cAMP compared with the adjacent construct.

Fig. 4. The effect of tonicity on the UT-A promoters. Promoter activity was induced by exposure of cells for 24 h to media made hypertonic (600 mosmol/kgH2O) by the addition of NaCl. Activity is shown as the ratio between firefly and Renilla luciferase activity/μg protein. Values are means ± SD; n = 3. **Significant increase in promoter activity compared with control, P < 0.01.

Therefore, UT-A promoter activity may be involved in regulating UT-Ab promoter in response to cAMP. Because no CREs were apparent in the UT-Aa promoter, progressive deletions were not engineered.

Analysis of the UT-Aa promoter region revealed a putative TonE/ORE consensus sequence at −315 bp. To determine whether either of the two mouse UT-A promoters was regulated by hypertonicity, MDCK cells transfected with UT-A constructs were grown in hypertonic media (600 mosmol/kgH2O) for 24 h. In a representative experiment (1 of 3), the UT-Aa-2.2 promoter activity in isotonic medium (0.060 ± 0.004 RLU/μg protein) significantly increased (P < 0.01) 1.6-fold (0.095 ± 0.010 RLU/μg protein) after exposure to hypertonic medium (Fig. 4). The 2.4-kb UT-Ab promoter construct, UT-Ab-2.4, which did not contain a TonE consensus sequence, showed no response to hypertonicity (n = 3). These data indicated that the activity of the UT-Aa promoter, but not the UT-Ab promoter, was modulated by tonicity.

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clude purine-rich splicing enhancers, which are usually located within exons, or pyrimidine-rich enhancers that reside within introns (16). In one class of purine-rich splicing enhancers, the nucleotide sequence of the 5′-splice site [AGGT(A/G)AGT] influences its splicing efficiency (4, 5). After bp 175 of exon 13, there is a consensus GT that acts as an intronic donor site for splicing of UT-A1. The flanking sequence of this site (TGGTGACT) does not exactly match the consensus. Base substitutions in flanking nucleotides, such as those apparent in exon 13, are known to weaken the influence this consensus motif has on splicing frequency. Thus, given the potential weakness of this site, we suggest that UT-A3 may be the default transcript resulting from activation of the UT-Aα promoter. Clearly, this remains speculative but provides an intriguing avenue for future research.

Overall comparison of the structure of the mouse UT-A gene with that of the rat (18) revealed that the structures of the two genes are very similar; the rat UT-A gene extends for greater than 300 kb and contains 24 exons. However, the transcript encoding UT-A5 has not been identified in the rat, although a candidate 1.7-kb transcript has been reported in rat testes (14). This suggests that at least one further exon remains to be identified in the rat gene. Similarly, the transcripts UT-A1b, UT-A2b, and UT-A3b have not been identified in the mouse, although the information to test for their existence is now available in the form of genomic sequence data we have generated.

Analysis of the two UT-A gene promoters revealed that UT-Aα is a TATA-less promoter and drives the transcription of UT-A1, UT-A3, and, possibly, UT-A4 from several start sites (Fenton RA, Stewart GS, Carpenter B, Howorth A, Cooper GJ, and Smith CP, unpublished observations). In contrast, the UT-Aβ promoter conforms to a classic TATA-box-containing promoter (13), and transcription of UT-A2 is initiated from a single locus (Fenton RA, Stewart GS, Carpenter B, Howorth A, Cooper GJ, and Smith CP, unpublished observations). The known mouse UT-A transcripts are therefore formed as a result of alternative pre-mRNA splicing, utilization of a split exon (exon 13) in combination with the use of an alternate polyadenylation signal, and differential promoter activity.

The arrangement of the mouse UT-Aα and UT-Aβ promoters is comparable to the two equivalent promoter regions identified in the rat (18, 19). As we have shown in the mouse, the rat UT-Aα promoter is also a TATA-less promoter and contains three CCAAT boxes, two GREs, and a single TonE/ORE (19). Furthermore, the rat UT-Aβ promoter, like its mouse counterpart, is a typical TATA-containing promoter and contains four CREs, compared with five found in mouse, and a single GRE (18).

Functional analysis of the two murine promoters using luciferase reporter constructs and reporter gene assays showed that both promoters initiate gene transcription and have a low basal level of promoter activity when transiently transfected into MDCK cells. We also found that the activity of both promoters was significantly enhanced by cAMP. Truncations of the mouse UT-Aβ promoter indicated that activation by cAMP was mediated by CREs. The CRE at −2,207 (see
Fig. 3) was found to have the greatest affect on promoter activity. In the rat, four CRE consensus sequences have been identified in the UT-Aβ promoter, and mutagenesis studies have confirmed that these CREs are responsible for mediating the response of the rat UT-Aβ promoter to cAMP (18). In agreement with these studies, our results strongly suggest that CRE consensus sequences in the UT-Aβ promoter are responsible for regulating the activity of UT-A2 expression in response to cAMP.

Intriguingly, we observed that the activity of the UT-Aα promoter, containing no CREs, was increased in response to cAMP. In addition to acting directly through CREs, cAMP can also increase transcription by interacting with other regulatory sequences such as activator protein (AP)-1 (6), AP-2 (20), Sp1 (31), and inverted CCAAT motifs (22). The UT-Aα promoter contains three AP-1 motifs, three Sp1 motifs, and four inverted CCAAT motifs; therefore, it is possible that UT-Aα promoter activity increases in response to cAMP because of an interaction between cAMP and one or more of these consensus motifs. In the rat, a 1.3-kb UT-Aα promoter construct showed no increase in transcriptional activity after stimulation by cAMP (18). This indicates that the mouse and rat UT-Aα promoters have different responses to cAMP, or, alternatively, that the element responsible for cAMP stimulation in the rat UT-Aα promoter is upstream of the sequence contained in the reporter construct analyzed in studies by Nakayama et al. (18).

The activity of the UT-Aα promoter, containing a consensus TonE/ORE regulatory sequence (12, 23), was significantly increased in response to hypertonicity, whereas increased toxicity had no effect on the activity of the UT-Aβ promoter that lacks a TonE/ORE motif. These findings agree with those reported by Nakayama and colleagues (19) for the rat UT-A promoters. Increasing toxicity, by the addition of NaCl, caused an increase in UT-Aa, but not UT-AB, promoter activity. As we discovered in the mouse, the rat UT-Aα promoter has a TonE/ORE motif, whereas the UT-Aβ promoter does not. Mutation of the rat TonE consensus abolished the response to hypertonicity, and gel-shift assays revealed that the TonE binding protein (17) binds to the rat UT-Aα promoter (19). In light of these findings, we conclude that the mouse UT-Aα promoter is responsive to changes in toxicity and suggest that this effect is mediated by a TonE consensus sequence.

Our analysis in the mouse and that of others in the rat (18, 19) suggested that both UT-A promoters are capable of responding to stimuli associated with antidiuresis, i.e., increased intracellular cAMP, mediated by vasopressin binding to the V2 vasopressin receptor, and elevated toxicity in the kidney medullary interstitium, caused by the action of the countercurrent multiplier. Therefore, we determined whether predicted increased activity of the UT-A promoters induced by fluid restriction led to commensurate changes in UT-A mRNA levels in vivo. Our analysis showed that mouse UT-A2 and UT-A3 mRNA levels increased after 20 h of fluid restriction. From this, we concluded that stimulation of the UT-A promoters by cAMP and of the UT-Aα promoter by increased medullary toxicity leads to an increase in UT-A2 and UT-A3 mRNA.

In summary, we have determined the genomic structure of the mouse UT-A gene and shown that activity of both promoters is increased by cAMP, whereas only UT-Aα promoter activity is increased by hypertonicity. We suggest that the increased levels of UT-A2 and UT-A3 mRNA, caused by fluid restriction, are a direct result of increases in UT-Aα and UT-Aβ promoter activity.

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REFERENCES