Cloning and Functional Characterization of a Rat Renal Organic Cation Transporter Isoform (rOCT1A)*

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Polyspecific organic cation transporters in the renal proximal tubule mediate the secretion of many clinically used drugs as well as endogenous metabolites. Recently, two organic cation transporters (rOCT1 and rOCT2) were cloned from rat kidney. In this study, we report the cloning and functional expression of an rOCT1 isoform, rOCT1A, from rat kidney. Genomic DNA cloning and sequencing demonstrated that rOCT1A is an alternatively spliced variant of rOCT1 with a deletion of 104 base pairs near the 5'-end. The uptake of [14C]tetraethylammonium (TEA) in oocytes injected with the cRNA-encoding rOCT1A was increased 16-fold over that in water-injected oocytes (29 ± 2.8 pmol/oocyte/h versus 1.8 ± 0.13 pmol/oocyte/h, mean ± S.E., p < 0.05). [14C]TEA uptake in the cRNA-injected oocytes was saturable (Km = 42 ± 11 μM) and was inhibited significantly by organic cations, including cimetidine and N1-methylnicotinamide. The amino acid sequence was deduced from the cDNA after examination of all three reading frames. Two overlapping open reading frames were found. Studies with synthetic constructs suggest that a functional organic cation transporter is encoded by the larger open reading frame. The larger open reading frame encodes a 430-amino acid protein (termed rOCT1A) that is 92% identical to rOCT1 and 57% identical to rOCT2. From hydrophathy analysis, rOCT1A is predicted to have 10 transmembrane domains with both amino and carboxyl termini intracellular. RNase protection assays demonstrate the presence of rOCT1A mRNA transcripts in rat kidney cortex, medulla, and intestine. These studies demonstrate the presence of a functional, alternatively spliced organic cation transporter (rOCT1A) in rat kidney.

* The abbreviations used are: TEA, tetraethylammonium; kb, kilo-base(s); bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; RPA, RNase protection assay; RF, reading frame; ORF, open reading frame; Pro, protein.

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lecular modeling, and studies with synthetic constructs suggest that a functional organic cation transporter (s) is encoded by the mRNA of rOCT1A after initiation of protein synthesis at an internal start codon. This is the first evidence of a functional, alternatively spliced variant of a polyspecific organic cation transporter.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning**—Total RNA was isolated from male Harlan Sprague Dawley rat kidneys and other tissues using TRIzol® reagent (Life Technologies, Inc.). Poly(A)+ RNA (mRNA) was selected by affinity chromatography using oligo(dT)-cellulose (American Life Science, Inc. 3 Prime, Inc., Boulder, CO). Total RNA or mRNA was primed with oligo(dT) primer to synthesize the first strand cDNA using the SuperPrime, Inc., Boulder, CO). Total RNA or mRNA was primed with oligo(dT) primer to synthesize the first strand cDNA using the SuperScript™ preamplification system for first strand cDNA synthesis (Life Technologies, Inc.). The synthesized cDNA and primers (10 μM) (see Table I and Fig. 1) specific for the rat kidney organic cation transporter (rOCT1) cDNA (17) were used in the subsequent PCR under the following conditions: 94 °C for 0.5 min, 55 °C for 1.5 min, 72 °C for 2 min, 35 cycles. The PCR products were electrophoresed through 1% agarose gels, and size-selected DNA fragments were extracted and subcloned into the pCR™II vector (original TA Cloning® kit, Invitrogen), or the pGEM-T vector (Promega) using T4 DNA ligase followed by transformation of DH5α (Life Technologies, Inc.) competent cells. Plasmid DNA was isolated using the Wizard™ miniprep DNA purification system (Promega) and was analyzed by restriction enzyme analysis and/or sequencing.

**Genomic DNA Cloning**—To obtain the genomic DNA sequence flanking the spliced region (exon/intron junctions), we used a novel method for walking upstream or downstream in genomic DNA from the cDNA sequence (26) with the Rat GenomeWalker™ kit (CLONTECH) according to manufacturer instructions. Primers were designed from the cDNA sequence upstream or downstream of the splice site (primers 5–8) (see Table I). PCR reactions were performed with the Advantage™ genomic PCR kit (CLONTECH) and used cycle parameters suggested by the manufacturer. The PCR products were subcloned as described above.

**Mutagenesis of rOCT1**—To generate the in-frame deletion (105 bp) variant of rOCT1, the QuickChange™ site-directed mutagenesis kit (Stratagene) was used according to manufacturer protocol. Briefly, two primers (sense and antisense to each other) flanking the mutagenesis site (primers 9 and 10, see Table I) were used in PCR with plasmid DNA containing the rOCT1A inserts as the template and Pfu DNA polymerase (Stratagene). The PCR product was then digested with DpnI restriction enzyme (Life Technologies, Inc.) followed by transformation and subcloning as described above.

**Sequence Analysis**—Subcloned cDNA inserts isolated from multiple reverse transcription and/or PCR reactions were sequenced using universal and gene-specific primers by the Biomedical Resource Center GenomicDNA Sequencing Facility at the University of California, San Francisco. Reverse transcription and/or PCR reactions were sequenced using universal and gene-specific primers by the Biomedical Resource Center GenomicDNA Sequencing Facility at the University of California, San Francisco. Forward and reverse primers were designed from the sequence (data not shown) (17). To determine whether the 1.5-kb cDNA encodes a functional organic cation transporter isoform (rOCT1), we obtained two PCR products of approximately 1.5 and 1.6 kb in size (Fig. 1B, lane 2). The PCR product at 1.6 kb matched the predicted size of the rOCT1 cDNA, whereas the 1.5-kb PCR product was of unknown origin. These two products were also detected after PCR using plasmid DNA isolated from a rat kidney cDNA library and the same primers (Fig. 1B, lane 3). Furthermore, both bands were detected from RT-PCR starting with total RNA isolated from the kidney of a single rat (data not shown). To confirm that the 1.5-kb PCR product was not generated from the mRNA transcript of the 1.6-kb PCR product due to either a PCR error or an error in reverse transcription, cDNA was isolated from the subcloned 1.6-kb PCR product and used in RT-PCR. This reaction resulted in a single detectable band on the gel (data not shown). Collectively, these data suggest that there are two RNA species in the rat kidney that can be amplified by RT-PCR using rOCT1-specific primers derived from the beginning and the end of the open reading frame (ORF) of the rOCT1 cDNA sequence. Furthermore, the resulting PCR product of primers 1 and 3 produced double bands (Fig. 1B, lane 5), whereas the product resulting from primers 2 and 4 produced a single band on the gel (Fig. 1B, lane 7), suggesting that the size of sequence is in close proximity to the 5′-end of the cDNA. Sequence analysis demonstrated that the sequence of the 1.6-kb cDNA was identical to the published rOCT1 cDNA sequence (data not shown) (17).

Functional Expression in Xenopus laevis Oocytes—To determine whether the 1.5-kb cDNA encodes a functional organic cation transporter isoform (rOCT1) in the Rat Kidney—Using first strand cDNA from mRNA isolated from several rat kidneys and primers 1 and 2 (Table I, Fig. 1A) derived from the published cDNA sequence of rOCT1 (17), we obtained two PCR products of approximately 1.5 and 1.6 kb in size (Fig. 1B, lane 2). The PCR product at 1.6 kb matched the predicted size of the rOCT1 cDNA, whereas the 1.5-kb PCR product was of unknown origin. These two products were also detected after PCR using plasmid DNA isolated from a rat kidney cDNA library and the same primers (Fig. 1B, lane 3). Furthermore, both bands were detected from RT-PCR starting with total RNA isolated from the kidney of a single rat (data not shown). To confirm that the 1.5-kb PCR product was not generated from the mRNA transcript of the 1.6-kb PCR product due to either a PCR error or an error in reverse transcription, cDNA was isolated from the subcloned 1.6-kb PCR product and used in RT-PCR. This reaction resulted in a single detectable band on the gel (data not shown). Collectively, these data suggest that there are two RNA species in the rat kidney that can be amplified by RT-PCR using rOCT1-specific primers derived from the beginning and the end of the open reading frame (ORF) of the rOCT1 cDNA sequence. Furthermore, the resulting PCR product of primers 1 and 3 produced double bands (Fig. 1B, lane 5), whereas the product resulting from primers 2 and 4 produced a single band on the gel (Fig. 1B, lane 7), suggesting that the product resulting from primers 2 and 4 produced a single band on the gel (Fig. 1B, lane 7), suggesting that the size of sequence is in close vicinity to the 5′-end of the cDNA. Sequence analysis demonstrated that the sequence of the 1.6-kb cDNA was identical to the published rOCT1 cDNA sequence (data not shown) (17).
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Sequences added that are not related to the rOCT1 cDNA, including restriction enzyme sites, are underlined, and mutation sites are indicated in italics.

| Sequence | Position |
|----------|----------|
| Primer 1 (sense)  | 5'-GCAGGGCTTGGGCTAAACTGGTAC-3' | 1-23 |
| Primer 2 (antisense) | 5'-TTTGGGCTCAGGTACTGTGGAG-3' | 1691-1708 |
| Primer 3 (antisense) | 5'-AGTGGGAACTGACGTC-3' | 1046-1060 |
| Primer 4 (sense) | 5'-ACGCGGCTTGCTGGGACGCGAG-3' | 887-904 |
| Primer 5 (sense) | 5'-ATGAGGCCCTGCGAGCATGGCTG-3' | 391-414 |
| Primer 6 (sense) | 5'-ACCCCACTGGCTGGTACACC-3' | 695-671 |
| Primer 7 (antisense) | 5'-AACATGGATGTATAGTCTGGG-3' | 648-628 |
| Primer 8 (antisense) | 5'-ATCGTCACTGAGTTTGGCC-3' | start at 440 |
| Primer 9 (sense) | 5'-GACGTCTGGCAACAATCTAAG-3' | start at 671 |
| Primer 10 (antisense) | 5'-GCCCTGGCTGGTGTTTAACCTGGTGTG-3' | 447-466 |
| Primer 11 (sense) | 5'-GGAATTCCTGGAATGGCATAGGCCA-3' | 801-817 |

A

rOCT1 cDNA

![Diagram showing the regions being amplified by RT-PCR with designated primers.](image)

B

![gel picture of RT-PCR.](image)

Fig. 1. Results of RT-PCR. A, diagram showing the regions being amplified by RT-PCR with designated primers. B, gel picture of RT-PCR. 1% agarose gel showing that two bands were detected from the RT-PCR products using rOCT1-specific primers derived from the beginning (primer 1) and the end (primer 2) of the ORF (lane 2) and the beginning (primer 1) and the middle (primer 3) of the ORF (first half) (lane 5), but a single band was detected when using primers from the middle (primer 4) and the end (primer 2) of the ORF (lane 7). Double bands also were detected when using plasmid DNA from a rat kidney cDNA library as a template and primers 1 and 2 (lane 3). Lane 1, 1-kb DNA ladder (from Life Technologies, Inc.); lanes 4, 6, and 8, no cDNA control; lane 9, no RT control.

To determine the specificity of transport, we studied the effect of various compounds on 

\[ ^{14}C \text{TEA} \]

uptake in the cRNA-injected oocytes. Consistent with the characteristics of polyspecific renal organic cation transporters, the organic cations (5 mM) TEA, guanidine, cimetidine, choline, \( N \)-methylnicotinamide, and procainamide all significantly inhibited the uptake of \( ^{14}C \text{TEA} \) (Fig. 2B, \( p < 0.05 \)). Similar functional characteristics of both rOCT1 and rOCT2 have been observed (17, 18). In addition, \( p \)-aminohippuric acid (5 mM), a model organic anion, weakly inhibited \( ^{14}C \text{TEA} \) uptake (Fig. 2B, \( p < 0.05 \)). Organic anions at high concentrations (e.g., 5 mM) have been shown previously to inhibit renal organic cation transporters (1, 29, 30). These data suggest that the 1.5-kb cDNA encodes a functional organic cation transporter having similar characteristics as rOCT1. Quantitative studies are under way to determine whether rOCT1 and rOCT1A differ in the potency of interaction with various substrates.

To determine the kinetic characteristics of TEA transport in oocytes injected with the cRNA of the 1.5-kb PCR product, we measured TEA uptake over a range of concentrations (30–500 \( \mu \text{M} \)). Fig. 2C shows the data along with the computer-generated nonlinear regression fit curve for the Michaelis-Menten equation. The \( V_{\text{max}} \) and \( K_m \) of TEA in this experiment were 5.4 ± 0.35 pmol/oocyte/h and 42 ± 11 \( \mu \text{M} \), respectively. The \( K_m \) of TEA uptake (42 \( \mu \text{M} \), Fig. 2C) is slightly lower than the value of 95 \( \mu \text{M} \) obtained previously for rOCT1 (17). The \( V_{\text{max}} \) is considerably lower (5.4 versus 81 pmol/oocyte/h (17)), suggesting that there may be fewer functional rOCT1A transporters present; however, \( V_{\text{max}} \) values are difficult to compare due to differences in experimental conditions between laboratories.

DNA Sequencing and Primary Amino Acid Sequence—After ascertaining the function of the transporter, we carried out sequence analysis to deduce the primary sequence of the functional protein from the cDNA sequence. Because PCR may result in fidelity errors in amplification of DNA, we performed sequence analyses of cDNAs isolated from multiple reverse transcription and PCR reactions. A consistent sequence was obtained (Fig. 3A). DNA sequence alignment between the 1.5- and 1.6-kb clone (rOCT1) demonstrates that the 1.5-kb cDNA sequence is identical to that of the 1.6-kb cDNA with a deletion between bp 451 and 556 of the rOCT1 cDNA. This is consistent with the PCR results (Fig. 1B). The deletion results in a stop codon at bp 460 in reading frame 2 (RF2, the same reading frame encoding the ORF of rOCT1), and a large ORF is present from the ATG at bp 312 to the stop codon at bp 1602 in RF3 (Fig. 3A). This large open reading frame in RF3 encodes a batch of oocytes (range approximately 2–20-fold).
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Genomic DNA Cloning—To determine the genomic nature of rOCT1A, genomic DNA fragments of rOCT1 flanking the splice sites were cloned and sequenced. As shown in Fig. 4, there is an intron of at least 6 kb in length between bp 451 and 452 of the rOCT1 cDNA followed by a short exon of 104 bp in length, which matches the deleted sequence. There is another intron of 439 bp in length between bp 555 and 556 of the rOCT1 cDNA. Based on the genomic sequence of rOCT1 in this region and the fact that rOCT1 and rOCT1A are 100% identical at the cDNA level (except for the 104-bp deletion in rOCT1A), rOCT1A represents an alternatively spliced isoform from a common precursor mRNA transcript. In addition, a recent chromosomal localization study of Roct1 excludes the possibility of multiple Roct1 genes or pseudogenes (32).

The 104 bp (exon B) in rOCT1 include part of the loop between transmembrane domain 1 and 2 and the whole transmembrane domain 2. This deletion interrupts a codon (AGG) between 555 and 556 that results in a frameshift and leads to an earlier stop codon in RF2. Frameshift in splice variants of membrane proteins in higher organisms (i.e. humans and other mammals) has been reported (19, 33, 34). In addition, the large intron of at least 6 kb between exon A and B presents the possibility of further alternative splicing. It will be of interest to ascertain whether other subtypes or isoforms of rOCT1 exist and to determine their functional significance.

In Vitro Translation—In vitro translation experiments in the rabbit reticulocyte lysate system were performed to synthesize proteins from the cRNAs of rOCT1A and rOCT1 (as a comparison). Translation products were analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 5 shows that translation of the cRNA of rOCT1 produced a single band with an apparent molecular size of 47 kDa (Fig. 5, lane 2). In contrast, translation of the mRNA encoding rOCT1A resulted in several bands with one major band of about 16 kDa in size and a major band(s) of about 37 kDa (Fig. 5, lane 3). The multiple bands at 37 kDa may be a result of translation beginning at the internal ATG at bp 312.) Additionally, one potential N-glycosylation site was identified in the extracellular loop between helices 7 and 8. These sites are conserved in both rOCT1 and rOCT1A (17).

protein (rOCT1A) of 430 amino acids that, after sequence alignment, is 92% identical to that of rOCT1 (the reading frame of rOCT1 is restored in the RF3 of rOCT1 cDNA after the deletion junction, and the only different region lies at the amino terminus) and 57% identical to that of rOCT2. (Alternative ATG sites at bp 408 or 540 of RF3 are other possible initiation sites and would encode truncated versions of rOCT1A.) Based upon hydropathy analysis using the Kyte-Doolittle algorithm and application of the positive inside rule (27, 31), rOCT1A is predicted to have 10 transmembrane domains with both amino and carboxyl termini intracellular (Fig. 3B). In comparison, both rOCT1 and rOCT2 are predicted to have 12 transmembrane domains (17, 18). As a result of the deletion, rOCT1A lacks the first two transmembrane domains as well as the three potential glycosylation sites in the first extracellular loop of rOCT1. Nevertheless, rOCT1A exhibits similar functional characteristics to those of rOCT1, which implies that the first two transmembrane domains and these three putative glycosylation sites are not essential for the transport function. However, other properties of the transporter such as synthesis, targeting, and sorting may be different between the two isoforms. Five potential protein kinase C phosphorylation sites at serine residues 160, 166, and 202 and threonine residues 170 and 424 were identified in the intracellular loops of rOCT1A. (The positions correspond to the deduced amino acid sequence of rOCT1A beginning at the internal ATG at bp 312.) Additionally, one potential N-glycosylation site was identified in the extracellular loop between helices 7 and 8. These sites are conserved in both rOCT1 and rOCT1A (17).

Fig. 2. Functional expression of rOCT1A in Xenopus laevis oocytes. A, [1^4C]TEA uptake in rOCT1 cRNA-injected oocytes compared with water-injected and mRNA-injected oocytes. [1^4C]TEA (500 μM) uptake by oocytes was assayed for 90 min at 25 °C 3 days after injection of 50 nl of water, rOCT1A cRNA, or rat total mRNA. Each column represents the mean ± S.E. of one representative experiment. Seven to nine oocytes were used for each column. Dark bars represent the uptake in the absence of 5 mM cimetidine, and open bars represent the uptake in the presence of 5 mM cimetidine. B, effect of various organic cations and the organic anion (ethylene isethionate (500 μM) uptake by oocytes injected with water or rOCT1A cRNA. Each column represents the mean ± S.E. of one representative experiment. Seven to nine oocytes were used for each column. NMN, N^3-methyl-N’-nicytosinamide; PAH, p-aminobenzopurpurate. All data were significantly different from the control, p < 0.001. Control represents uptake in oocytes in the absence of unlabeled compounds. C, kinetics of TEA transport in rOCT1A cRNA-injected oocytes. Data were fit to a Michaelis-Menten equation (K_m = 42 ± 11 μM; V_max = 5.4 ± 0.35 pmol/oocyte/h). Each point represents mean ± S.E. from seven oocytes.
products produced in the in vitro translation experiments have apparent molecular masses smaller than predicted based upon the deduced sequences. However, anomalous migration of membrane proteins is not unusual (35, 36).

To determine whether the smaller band of about 16 kDa was due to the early stop codon at 460 bp in RF2, a mutant of rOCT1A was generated. This mutant contained a 105-bp in-frame deletion in the rOCT1 cDNA, thus eliminating the early stop codon and restoring the RF2 as in rOCT1 of the spliced RNA. As shown in Fig. 5, in vitro translation of the cRNA of this mutant produced a single band (lane 4). These data provide the indirect evidence suggesting that the 16-kDa band observed after in vitro translation of the cRNA of rOCT1A may be encoded by the short ORF in RF2 of rOCT1A. In addition, after in vitro translation, there is a notable size difference in the larger band from the cRNA of the mutant in comparison with the larger band(s) from the cRNA of rOCT1A. Such a size difference would not be expected with the difference in the length of the cDNA's encoding rOCT1A and the mutant, i.e. 1 bp.

Many naturally occurring cellular and viral mRNAs are polycistronic, and multiple proteins are synthesized by re-initiation of translation at internal AUGs after meeting the terminator codon of the upstream ORF. Examples of polycistronic mRNA transcripts in eukaryotes are rare and have never been reported in mammalian mRNA (37–39). Further studies are needed to determine whether the multiple bands observed in in vitro translation studies are a result of a polycistronic mRNA transcript of rOCT1A or proteolysis. Future studies utilizing antibodies recognizing different ORFs will provide the definitive evidence to determine whether multiple proteins from different ORFs were encoded. Mutagenesis studies will be important in determining the internal initiation site(s) of translation for rOCT1A.

FIG. 3. A, cDNA sequence of the 1.5-kb (rOCT1A) clone and the corresponding three possible translational reading frames. The positions of the four putative initiation sites are indicated in bold. Nucleotides flanking the splice sites are indicated by a bold underline. The putative amino acid sequence of rOCT1A is underlined. Stop codons are indicated by asterisks. B, Kyte-Doolittle hydrophathy analysis of rOCT1A (the protein encoded from bp 312 to bp 1601) using a window of 11 amino acids; putative transmembrane domains are numbered (1–10).
We hypothesized that the larger protein is the functional transporter. Accordingly, we constructed a synthetic cDNA that would encode for the 16-kDa product (Pro 1) and two synthetic cDNAs that would encode for two possible functional transport proteins (Pro 2 and Pro 3) (Fig. 6A). The function of the synthetic proteins was tested by injecting oocytes with cRNA transcribed from the cDNA encoding Pro 1, 2, and 3. [14C]TEA uptake was enhanced significantly in oocytes injected with the cRNA of Pro 2 or 3 in comparison to water-injected oocytes (Fig. 6B, p < 0.05). In contrast, [14C]TEA uptake was not enhanced in oocytes injected with the cRNA of Pro 1 (2.35 ± 0.13 pmol/oocyte/h in cRNA-injected oocytes versus 2.51 ± 0.31 pmol/oocyte/h in water-injected oocytes, mean ± S.E.). The reasons why Pro 3 had a higher expressed activity than either Pro 2, rOCT1, or rOCT1A are unknown. Differences in efficiency of the in vivo translation or protein processing may explain the data. Alternatively, Pro 3 may have a higher intrinsic activity. These data suggest that Pro 2 and 3, representing possible proteins encoded by the cRNA of rOCT1A, can mediate the transport of TEA, whereas Pro 1 cannot. In in vitro translation experiments, Pro 2 migrated at a similar rate as the 37-kDa translation product of the cRNA of rOCT1A (data not shown), suggesting that Pro 2 might be the functional organic cation transporter. These data have implications to the function of rOCT1, since rOCT1A is 92% identical in sequence to rOCT1.

RNA Expression of rOCT1A and Its Tissue Distribution—To characterize the rOCT1A RNA expression in the kidney, RPAs were performed. A 416-nucleotide biotin-labeled antisense RNA probe was synthesized to specifically detect rOCT1 and rOCT1A RNA fragments differing by approximately 110 nucleotides (Fig. 7A). As shown in Fig. 7B, two RNAase-protected fragments of expected size for rOCT1 and rOCT1A (370 and 263 nucleotides, respectively) were detected in the RNA of total kidney, kidney cortex, and kidney medulla. In addition, from the intensities of the protected fragments, it appears that the rOCT1 RNA transcript is present at a higher level than that of rOCT1A in the kidney.
Although the mRNA transcript of rOCT1A and rOCT1 is similar but differs from that of rOCT2, RT-PCR indicates that rOCT1A mRNA transcripts are also present in the intestine and liver, and RNase protection assays indicate that the RNA transcript of rOCT1A is present in detectable quantities in the rat kidney. Future studies will be performed to determine the underlying structural requirements for the function of organic cation transporters, the physiologic role of rOCT1A in the intact tissue, and the mechanisms involved in the translation of the rOCT1A mRNA transcript.

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