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Short sequence paper

Molecular cloning and functional expression of a rabbit renal organic cation transporter [1]

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Abstract

A cDNA encoding an organic cation transporter (rbOCT1) was isolated from rabbit kidney. The cDNA encodes a 554 amino acid protein that is highly homologous to other mammalian organic cation transporters. rbOCT1 mediated ^{3}H-1-methyl-4-phenylpyridinium (^{3}H-MPP^{+}) transport in *Xenopus laevis* oocytes was saturable, sensitive to membrane potential, and inhibited by various organic cations. rbOCT1 mRNA transcripts are expressed in the kidney, liver, and intestine. © 1998 Elsevier Science B.V.

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Active secretion in the renal proximal tubule is a major route of elimination of many organic cations from the systemic circulation. Tubule secretion of organic cations in the rabbit involves at least two distinct steps [1]. In the first step, organic cations move from the blood into the intracellular space via a potential sensitive or an electroneutral exchange mechanism in the basolateral membrane. In the second step, organic cations are transported across the apical membrane and into the tubule lumen by an electoneutral organic cation–proton exchange mechanism(s).

The mechanisms of renal organic cation transport have been studied extensively in various experimental preparations from rabbit kidney including perfused [2,3] and nonperfused [2,4–7] tubules, apical [8–10] and basolateral [6,11] membrane vesicles, and isolated tissue slices [12]. Collectively, results from many studies suggest that multiple organic cation transporters are present in both membranes of the renal proximal tubule. For example, there is evidence that tetraethylammonium (TEA) and N^{1}-methylnicotinamide (NMN) are transported across the basolateral membrane by distinct transporters. Namely, in nonperfused tubules, the relative accumulation of TEA is equal in all three proximal tubule segments whereas the relative accumulation of NMN is highest in S2 and S3 segments and is much lower in the S1 segement [2,5]. Both an electroneutral organic cation–organic cation exchange mechanism and a

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potential sensitive organic cation transport mechanism have been characterized in rabbit renal basolateral membrane vesicles. It has been hypothesized that these two transport mechanisms are the result of a single transporter operating in two modes [11].

The first organic cation transporter was cloned from a rat kidney cDNA library by expression cloning [13]. Subsequently other organic cation transporters have been cloned from rat [14,15], human [16], and pig [17]. The overall two-step model of renal organic cation transport appears to be conserved among various species based on reports in the literature [1]. However, notable interspecies differences have been reported at the mechanistic and molecular level [4,16]. For example, a recent study showed a significant species difference between rat and rabbit in the interaction of *n*-tetraalkylammonium compounds with renal organic cation transport systems [4].

To understand the multiple transport mechanisms present in the rabbit kidney and to investigate the underlying cause(s) of the observed interspecies differences in organic cation transport the molecular cloning of rabbit renal organic cation transporters is essential. Here we report the cloning and functional expression of the first organic cation transporter from rabbit kidney.

Homology based PCR and Rapid Amplification of cDNA Ends (RACE) PCR methods were used to obtain a full length cDNA from rabbit kidney. Total RNA was extracted from tissues of adult male New Zealand White rabbits using TriZOL reagent (Life Technologies). Poly(A)$^+$ RNA was purified by oligo(dT) cellulose affinity column chromatography. The cDNA template for PCR was synthesized from isolated kidney mRNA with the oligo(dT) primer using the SuperScript Preamplification System (Gibco-BRL, USA). Primers 1 and 2 (Table 1) were designed from conserved regions of other mammalian organic cation transporters. The PCR was performed in a thermal cycler (Perkin Elmer, USA) according to the following program: 94°C for 1 min, 48°C for 1 min 50 s, and 72°C for 3 min for 40 cycles followed by a final 15 min incubation at 72°C. PCR products were subcloned and analyzed by restriction enzyme analysis and/or sequencing. To obtain the remaining 5′-portion of rabbit kidney OCT1 cDNA, a PCR-based method, 5′-RACE, was utilized. Three gene specific antisense primers (GSP), designated 5′-GSP-1, 5′-GSP-2 and 5′-GSP-3 (Table 1), were designed from the partial rbOCT1 sequence. First-strand cDNA synthesis for 5′-RACE was primed with 5′-GSP-1 (Table 1) using the 5′-RACE System (Gibco-BRL, USA) from isolated rabbit kidney total RNA. Homopolymeric tails were added to the 3′-Ends of the cDNA which was then amplified by PCR using the anchor primer 1 (Table 1) and nested 5′-GSP-2 primer (Table 1) for 40 cycles according to the following program: 94°C for 1 min, 55°C for 2 min, 72°C for 2 min. A nested PCR was performed with the anchor primer 2 (Table 1) and nested 5′-GSP-3 primer (Table 1) with

Table 1
Primers used for PCR cloning of rbOCT1 cDNA

Primer designation	Primer sequence
Primer 1	5′-AGCCATGCCCACCGTGGATGATGTC-3′
Primer 2	5′-GAATTCCACCAGAGAAGAATAAAAGAAGTCC-3′
5′-GSP-1	5′-AGCCGGGCGTGTCATACACCCA-3′
5′-GSP-2	5′-AGCGGCAGGTGGCTCCTGTTGG-3′
5′-GSP-3	5′-CACTGTATAGTTCAGCTCCTCC-3′
Anchor primer 1 [a] (5′-RACE)	5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′
Anchor primer 2 [a] (5′-RACE)	5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′
3′-GSP-1	5′-TACTACTGGTGTGTGCCGGAG-3′
3′-GSP-2	5′-TCGCTGGCAGACCTGTTCCG-3′
Adapter primer [b] (3′-RACE)	5′-AGATGAATTCAAGCTTAGGTACCAGTTTTTTTTTTTTTTTT-3′
Amplification primer [b] (3′-RACE)	5′-AGATGAATTCAAGCTTAGGTACCAGT-3′
5′-End primer	5′-TTAGCCCAGAGCAGGCCGGCCGAG-3′
3′-End primer	5′-AAGCGCCGTGTTCCAAAAAAGTTCCT-3′

[a] These primers were provided with the 5′- and 3′-RACE system (GIBCO BRL).
[b] These primers were modified from Ref. [23].

a different annealing temperature (50°C) to assess the specificity of the PCR product. 3′-RACE was used to obtain the remaining 3′-portion of rabbit kidney OCT1 cDNA. Two gene specific sense primers, designated 3′-GSP-1 and 3′-GSP-2 (Table 1), were designed from the partial rabbit kidney OCT1 sequence (the sequenced PCR fragment). First-strand cDNA synthesis was initiated at the poly(A)$^+$ tail of isolated rabbit kidney mRNA using the adapter primer (Table 1) in the 3′-RACE kit (GIBCO BRL). PCR was performed on this first-strand cDNA using the 3′-GSP-1 primer (Table 1) and the nested amplification primer (Table 1) with an annealing temperature of 50°C. The resulting amplified products from the nested PCR using the 3′-GSP-2 primer and the amplification primer (Table 1) under the same conditions were then subcloned as described above. The entire rabbit kidney OCT1 cDNA coding region was obtained with the 5′-End primer and 3′-End primer (Table 1), which were designed from the beginning and the end regions of the open reading frame according to the following PCR protocol: 94°C for 1 min, 55°C for 2 min, 72°C for 2 min for 35 cycles followed by a final 30 min incubation at 72°C. rbOCT1 cDNA was subcloned into a pGEM-T vector (Promega, Madison, WI) and was then transformed into DH5alpha competent cells.

cDNAs isolated from multiple reverse-transcription and PCR reactions were sequenced by the Biomolecular Resource Center DNA Sequence Facility at the University of California, San Francisco with an automated sequencer (Applied Biosystems, Model 373A). Gap and Bestfit programs in the Genetics Computer Group (Wisconsin Package, Version 8) software package as well as the SeqVu program (Version 1.0.1, James Gardner, The Garvan Institute of Medical Research, Australia) were used for multiple sequence alignments. To determine potential protein kinase C phosphorylation and *N*-glycosylation sites, the Motifs program in the Genetics Computer Group (GCG) package was used. The transmembrane domains of rbOCT1 were predicted based on hydropathy analysis using the Kyte-Doolittle algorithm [18], using a window of 11, in the GCG's Pepplot program.

rbOCT1 was expressed in *Xenopus laevis* oocytes for functional characterization as described previously [16]. Stage V and VI oocytes were injected with 25 ng of capped cRNA transcribed in vitro with T7 RNA polymerase (mCAP RNA Capping Kit; Strategene) from linearized plasmid DNA. The injected oocytes were maintained in modified Barth's solution at 18°C until use. The uptake of ^{3}H-MPP$^+$ (79.9 Ci/mmol, DuPont-New England Nuclear, Boston, MA) in oocytes was measured as described previously [16]. Briefly, groups of six to nine oocytes were incubated in uptake buffer containing MPP$^+$ (0.2 μM ^{3}H-MPP$^+$ and 0.8 μM unlabeled MPP$^+$) at 25°C. The incubation time for uptake measurements was 90 min; ^{3}H-MPP$^+$ uptake was linear up to 120 min (data not shown). Unlabeled compounds were added to the reaction mixture for inhibition studies. For the Michaelis-Menten study, ^{3}H-MPP$^+$ (0.15 μM) with various amounts of unlabeled MPP$^+$ were included in the reaction mixture. The resting membrane potentials of water and cRNA injected oocytes in depolarizing and physiologic buffers were determined as described previously [16].

RT–PCR with total RNA isolated from various tissues of adult male New Zealand White rabbits was used to assess tissue distribution of the mRNA transcript of rbOCT1. Briefly, total RNA was isolated from the tissues and subjected to RT–PCR with the 5′-End primer and 3′-End primer (Table 1) as described above. The PCR products were electrophoresed through a 1% agarose gel.

An amplification product of about 1.2 kb in length was obtained when the PCR was performed with Primers 1 and 2 and with rabbit kidney first-strand cDNA as the template. DNA sequencing revealed that the PCR product was 1165 bp in length and was 82% identical to rOCT1 cDNA suggesting that a partial cDNA encoding a rabbit organic cation transporter had been obtained. To clone the full-length cDNA a PCR-based cloning strategy involving 5′- and 3′-RACE protocols was pursued. The resulting 5′-(~ 300 bp) and 3′-(~ 1700 bp) RACE products showed overlap with the 1165 bp cDNA isolated above as indicated by sequence analysis. The full-length cDNA was obtained by RT–PCR using primers flanking the 5′- and 3′-Ends and rabbit kidney mRNA as the initial template and designated rbOCT1.

The full length cDNA is 2732 bp and contains a 55 bp 5′-untranslated region (UTR), a 1665 bp open reading frame (ORF), and a 1012 bp 3′UTR (Fig. 1). The 1665 bp ORF is predicted to encode a 554 amino acid protein, whose initiation codon is in a Kozak

consensus sequence, A/GXXAUG [19]. Based upon Kyte-Doolittle hydropathy analysis [18], 12 transmembrane domains are predicted. The protein sequence contains three potential N-linked glycosylation sites (N-X-T/S) at positions 71, 96, and 112. In addition, three potential protein kinase C (PKC) phosphorylation sites were identified at residues 285, 291, and 327, which may be important in the regulation of rbOCT1. There has been one report in the literature which suggests that protein kinase C plays a role in the regulation of organic cation transporter(s) in the S2 segment of the rabbit nephron [20]. With the cloning of rbOCT1, the role of PKC in the regulation of this transporter can now be ascertained. Multiple sequence alignment of the rbOCT1 protein with other members of the gene family showed that the overall positions of secondary structure elements (e.g. transmembrane domains and loops) are well conserved; however, differences in N-linked glycosylation and protein kinase C sites were observed. BLAST searches of the protein and gene databases indicated that the rbOCT1 protein belongs to a growing number of related transporters found in mammals and other organisms. rbOCT1 is most homologous to other mammalian organic cation transporters: rOCT1 [13] (91% similarity and 81% identity), Lx1 [21] (the putative murine homolog of rOCT1) (85% similarity and 81% identity), hOCT1 [16] (83% similarity and 80% identity), rOCT2 [15] (72% similarity and 65% identity), and OCT2p [17] (75% similarity and 69% identity). In addition, it shares significant homology with recently cloned genes from the nematode *C. elegans* (42% similarity and 32% identity; GenBank accession number Z83228) and from the fruit fly *Drosophila* (46% similarity and 36% identity; GenBank accession number Y12400).

In *Xenopus laevis* oocytes injected with the cRNA of rbOCT1, the uptake of the model organic cation, ^{3}H-MPP^{+}, was enhanced 25-fold over that in water-injected oocytes four days post injection (Fig. 2(A)).

Fig. 1. Nucleotide and deduced amino acid sequence of rbOCT1. The start (ATG) and stop (TAA) codons are indicated in bold. The 12 putative transmembrane domains are underlined. Potential *N*-glycosylation sites of the type N-X-T/S are indicated with asterisks. Potential protein kinase C phosphorylation sites are indicated by points.

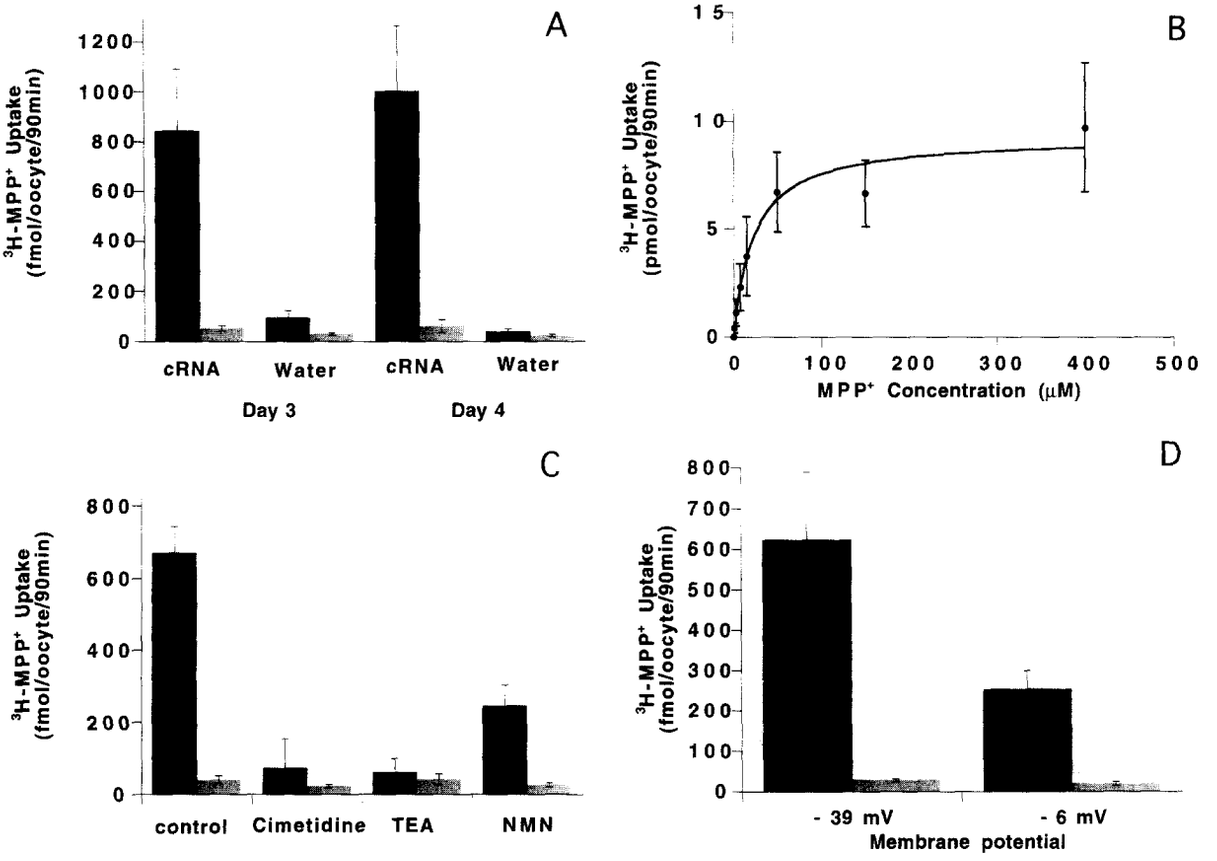

Fig. 2. Functional expression of rbOCT1 in *Xenopus laevis* oocytes. (A) ^{3}H-MPP$^+$ uptake in cRNA and water-injected oocytes in the presence (grey bars) or absence (dark bars) of 5 mM cimetidine three and four days post injection. (B) Kinetics of MPP$^+$ transport in rbOCT1 cRNA-injected oocytes. Apparent K_m and V_{max} values were determined by fitting to the Michaelis-Menten equation by non-linear regression analysis (Kaleidgraph, Abelbeck Software). (C) Inhibition of ^{3}H-MPP$^+$ uptake in rbOCT1 cRNA-injected (dark bars) and water-injected (grey bars) oocytes by model organic cations (5 mM). Control represents uptake in the absence of inhibitors. (D) Effect of membrane potential on ^{3}H-MPP$^+$ uptake in cRNA (dark bars) and water-injected (grey bars) oocytes. Values are means ± S.D.

The rate of MPP$^+$ uptake was saturable (Fig. 2(B)) with a K_m value of 23 ± 6 μM and a V_{max} of 9.25 ± 0.66 pmol/oocyte/90 min. The organic cations, cimetidine, TEA, and NMN, significantly inhibited the uptake of ^{3}H-MPP$^+$ (Fig. 2(C)). However, TEA appears to be a more potent inhibitor than NMN. Uptake in cRNA-injected oocytes was dramatically influenced by the membrane potential of the oocytes (Fig. 2(D)). In depolarizing buffer (2 mM NaCl, 100 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.4), in which the membrane potential was −6.4 ± 0.6 mV, the uptake of ^{3}H-MPP$^+$ in cRNA-injected oocytes was 60% lower compared to uptake in oocytes in a physiologic buffer (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.4), in which the membrane potential was −38.7 ± 2.2 mV. This result suggests that rbOCT1 may be localized to the basolateral membrane. As stated earlier, potential sensitive organic cation transport occurs at the basolateral membrane of the rabbit proximal tubule.

RT–PCR and Northern blot analysis were used to determine the tissue distribution of rbOCT1 mRNA transcripts. rbOCT1 was cloned from rabbit kidney, and RT–PCR analysis showed that its expression is confined to the cortex in the kidney. In addition, rbOCT1 transcripts were detected in the liver and intestine (Fig. 3). Based on band intensities, it appears that rbOCT1 is expressed at highest levels in the liver and to a lesser extent in the kidney cortex

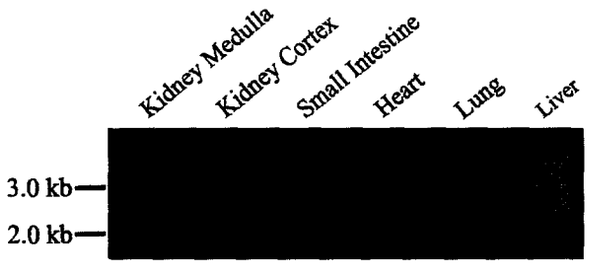

Fig. 3. RT–PCR analysis of rbOCT1 mRNA transcript expression. Total RNA from rabbit tissues was subjected to RT–PCR using primers 5′-End primer and 3′-End primer (Table 1). The RT–PCR products were analyzed by agarose gel electrophoresis. A molecular weight standard was used to determine the band size.

and intestine. Northern analysis of mRNA derived from various rabbit tissues (including brain, heart, lung, kidney cortex, kidney medulla, intestine, and liver) with a full-length rbOCT1 antisense cRNA probe only detected transcripts in the liver. Two transcripts produced signals of equal intensities at ~2.7 and ~4.4 kb (data not shown). Hence, the results of Northern analysis are consistent with the RT–PCR analysis, namely, that rbOCT1 transcripts are expressed most abundantly in the liver and at lower levels in other tissues. The low level of rbOCT1 expression in the kidney is somewhat surprising. This may indicate that other transporters play a greater role in organic cation transport in the rabbit kidney. Alternatively, rbOCT1 may be expressed in only a specific segment of the nephron where it would mediate site specific transport of organic cations. It should be noted that other renal transporters (e.g. the human PEPT2) have been reported to be expressed at low levels and were also undetectable by Northern blot analysis [22].

In summary, we have cloned and functionally expressed the first organic cation transporter from rabbit kidney (rbOCT1). Because much of our understanding of the mechanisms of renal organic cation transport is based primarily upon studies performed in rabbit kidney, the cloning of rbOCT1 represents an essential step in elucidating the mechanisms of organic cation transport in the kidney at a molecular level. In addition, notable interspecies differences in the interaction of bulkier organic cations with the respective transporters in rat and rabbit kidney have been observed [4]. With the availability of rbOCT1 and rOCT1, we can now carry out detailed kinetic studies to elucidate possible interspecies differences in the function of the two transporters.

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