Table 1. Primer sets and probes for real-time PCR and RT-PCR[a]

Gene Name (Accession)	Positions[c]	Sequences (5′ to 3′)
For real-time PCR[b]		
hMATE1 (AK001709)		
forward primer (+)	1240 to 1262	ATTGGGTACTATGTGGTTGGCCT
reverse primer (−)	1333 to 1311	AGATGATGATCCCTGACCACAGA
TaqMan probe (+)	1273 to 1298	ATCGCGCTGATGTTTGCAACCACACT
hMATE2 (NM_152908)[d]		
forward primer (+)	1444 to 1467	ACTGCTGCCTTTGTTGCTTATACT
reverse primer (−)	1570 to 1551	TCTCAGGCCCAGGTCTGGTT
TaqMan probe (−)	1541 to 1519	TCTGCTCTCTGCTGCTGCTGCCG
For RT-PCR		
forward primer (+)		ATCGTGAGCACTGTGTTCTGC
hMATE2-K (AB250364)	155 to 177	
hMATE2-B (AB250701)	155 to 177	
hMATE2 (NM_152908)	155 to 177	
reverse primer (−)		TGGGAGATGATGTTGGCATA
hMATE2-K (AB250364)	659 to 640	
hMATE2-B (AB250701)	813 to 794	
hMATE2 (NM_152908)	767 to 748	
hGAPDH (NM_002046)		
forward primer (+)	24 to 44	CAACGGATTTGGTCGTATTGG
reverse primer (−)	836 to 816	TGCTCAGTGTAGCCCAGGATG

[a]hMATE1, human multidrug and toxin extrusion 1; hMATE2-B, human brain-specific multidrug and toxin extrusion 2; hMATE2-K, human kidney-specific multidrug and toxin extrusion 2; RT-PCR, reverse transcriptase–PCR.
[b]Directions of the primer sequences are denoted in the parentheses as sense (+) or antisense (−).
[c]The nucleotides are from the sequence in the GenBank Data Bank and are numbered starting at the first residue of ATG putative initiation codon.
[d]Although only the nucleotide numbers for hMATE2 are shown, the primer and probe set also reacts with hMATE2-K and hMATE2-B.

according to the method of Musfeld *et al.* (19). For manipulation of the intracellular pH, intracellular acidification was performed by pretreatment with ammonium chloride (30 mM, 20 min at 37°C, pH 7.4) (20,21). The protein content of the solubilized cells was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as a standard.

Statistical Analyses

Data are expressed as the mean ± SEM. Data were analyzed statistically using an unpaired *t* test. Significance was set at $P < 0.05$. In all figures, when error bars are not shown, they are smaller than the symbols.

Results

After the sequencing of hMATE2 cDNA that was isolated from the human kidney (hMATE2-K) and human brain (hMATE2-B), it was revealed with the BLAST program that both hMATE2-K and hMATE2-B transcripts consist of 17 exons. However, a deletion of 108 bp in exon 7 of hMATE2-K and an insertion of 46 bp in exon 7 of hMATE2-B were found compared with hMATE2 (Figure 1). The open reading frame of the cloned hMATE2-K cDNA was 1698 bp, coding for a 566–amino acid protein with a calculated molecular mass of 61,012, and that of hMATE2-B was 660 bp and a 220–amino acid protein with a calculated molecular mass of 23,357. Figure 2 shows the deduced amino acid sequences of hMATE2-K and its alignment with its homologues hMATE2-B, hMATE2, hMATE1, or rat (r) MATE1, which was cloned recently in our laboratory from rat kidney with an accession no. AB248823 (22). hMATE2-K showed 82% amino acid identity with hMATE2-B, 94% with hMATE2 (12), 52% with hMATE1 (12), and 52% with rMATE1.

The hMATE1 mRNA was expressed strongly in the adrenal gland as well as in the kidney; weakly in the fetal liver, testis, skeletal muscle, liver, and uterus; and faintly in various other tissues (Figure 3A). In contrast, the obtained data showed that the transcript of the *hMATE2* gene was expressed in the kidney, because the real-time PCR condition could cross-react hMATE2-K, hMATE2-B, and hMATE2 (Figure 3A, Table 1). Although RT-PCR showed that the amplification of both products derived from hMATE2-K (505 bp) and hMATE2-B (659 bp) among 18 tissues examined, only the 505-bp band that corresponded to hMATE2-K was found in the kidney (Figure 3B). The 613-bp band that corresponded to hMATE2 was not amplified. After sequencing, the PCR product of 659 bp was confirmed to be identical to hMATE2-B.

To visualize the intrarenal distribution of hMATE2-K, we examined the immunohistochemical analysis (Figure 4). Staining with the antibodies specific for hMATE1 and hMATE2-K revealed that both transporters were localized in the brush

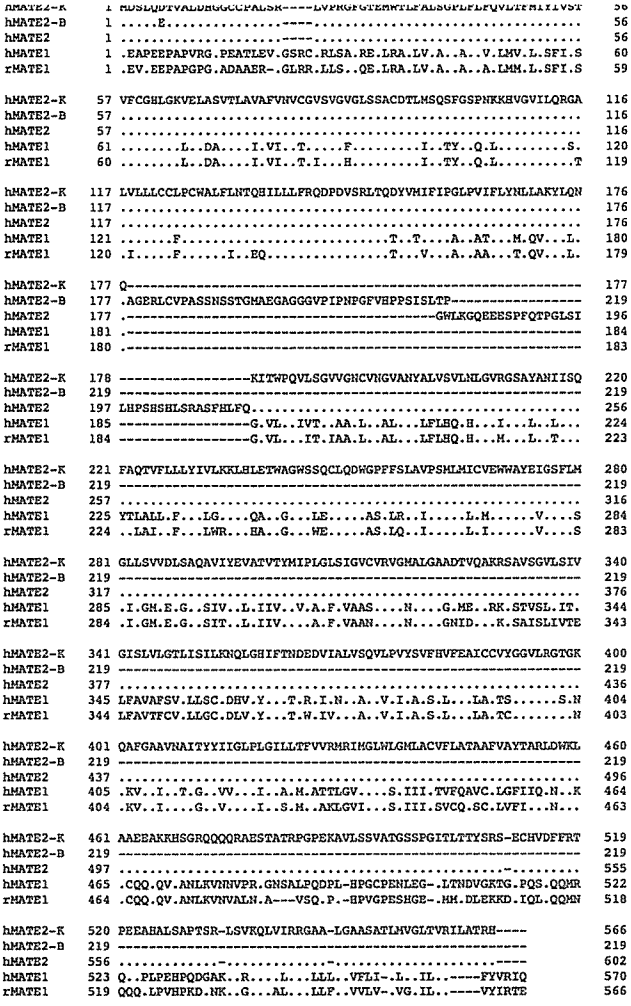

Figure 2. Comparison of the deduced amino acid sequences among hMATE2-K, hMATE2-B, hMATE2, hMATE1, and rat multidrug and toxin extrusion 1 (rMATE1). The conserved residues in hMATE2-K are indicated by dots. The accession numbers for hMATE2-K, hMATE2-B, hMATE2, hMATE1, and rMATE1 are AB250364, AB250701, NM_152908, AK001709, and AB248823, respectively.

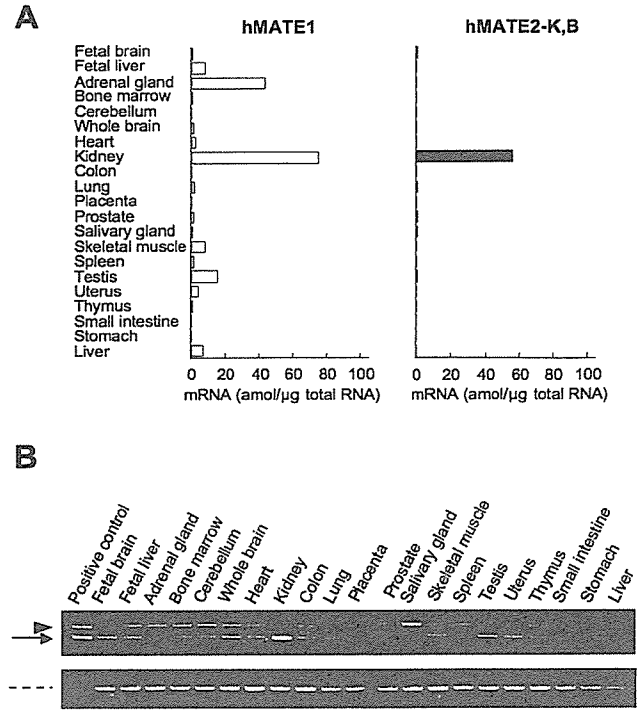

Figure 3. Real-time PCR for hMATE1 and hMATE2 (A), and the splice variant-specific reverse transcriptase–PCR (RT-PCR) analysis for hMATE2-K and hMATE2-B (B). (A) Total RNA isolated from various human tissues were reverse transcribed, and mRNA levels of hMATE1 and hMATE2 with their spliced variants were determined by real-time PCR with the oligonucleotides summarized in Table 1 using an ABI PRISM 7700 sequence detector. The mRNA expression level of hMATE1 and hMATE2 was calculated by the absolute standard method as described (13). As described in the legend for Table 1, the primer and probe set for hMATE2 cross-reacts with both hMATE2-K and hMATE2-B; therefore, the data are the mRNA levels of hMATE2, hMATE2-K, and hMATE2-B. Each column represents the mean of two separate experiments. (B) The RT-PCR amplification of hMATE2-K and hMATE2-B mRNA with the primer sets covering exon 7 of each gene shown in Table 1. The PCR products of 505 bp that corresponded to hMATE2-K (arrow) and 659 bp that corresponded to hMATE2-B (arrowhead) were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. No signal was observed of 613 bp that corresponded to hMATE2. The quality of the sample RNA also was checked by RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (dotted line). The mixture of plasmid DNA coding hMATE2-K and hMATE2-B was used as a positive control for hMATE2-K and hMATE2-B and as a negative control for GAPDH.

border membranes of proximal tubules (Figure 4, A through F). Although the green signals for F-actin with phalloidin were shown in all sections, red signal was not observed in the section stained with the preimmune serum (Figure 4G). The red signals for hOCT2, which is a basolateral organic cation transporter, and the hOCTN2, which is a luminal Na^+/carnitine co-transporter, were confirmed in the basolateral and brush border membranes, respectively (Figure 4, H and I).

The oppositely directed H^+ gradient–dependent uptake of [^{14}C]TEA was examined in HEK293 cells that were transfected with the pcDNA3.1 (+) empty vector, hMATE2-K cDNA, and hMATE2-B cDNA. The pH gradient–dependent uptake of [^{14}C]TEA was stimulated in cells that were transfected with hMATE2-K cDNA but not with hMATE2-B cDNA (Figure 5A). In addition, intracellular acidification by ammonium chloride markedly stimulated the hMATE2-K–mediated uptake of [^{14}C]TEA, but no response was observed in hMATE2-B–transfected cells (Figure 5B). The hMATE2-K–mediated [^{14}C]TEA uptake was not affected by co-transfection of hMATE2-B cDNA (Figure 6). Therefore, the protein that was coded by hMATE2-B seemed to have no influence on the activity of hMATE2-K or its

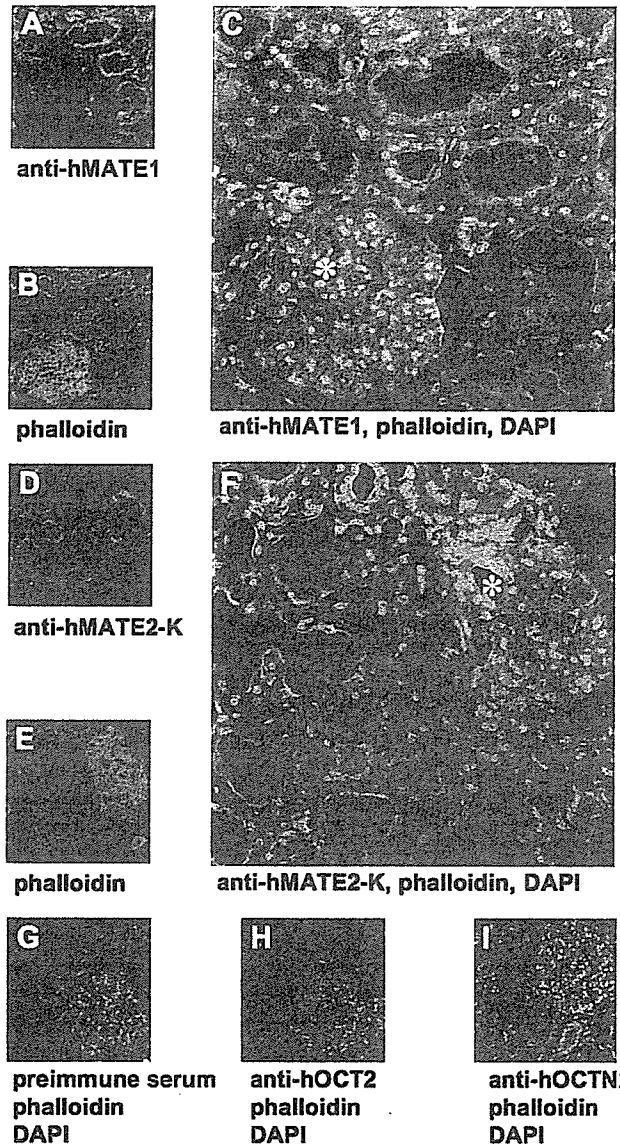

Figure 4. Immunofluorescence localization of hMATE1 and hMATE2-K in the human kidney. The hMATE1 (red; A), F-actin (green; B), and merged picture including the purple signals of 4′,6-diamidino-2-phenylindole (DAPI; C) were observed in the same section. The hMATE2-K (red; D), F-actin (green; E), and merged picture including DAPI (F) were in the same section. The yellow signals consist of hMATE1 or hMATE2-K and F-actin and were concentrated in the brush border membranes of proximal tubules (C and F). No positive staining for hMATE2-K (red) was observed using the preimmune serum (G). The basolateral localization of hOCT2 (H) and the apical localization of hOCTN2 (I) were confirmed with each antibody, respectively. *Glomeruli. Magnification, ×100.

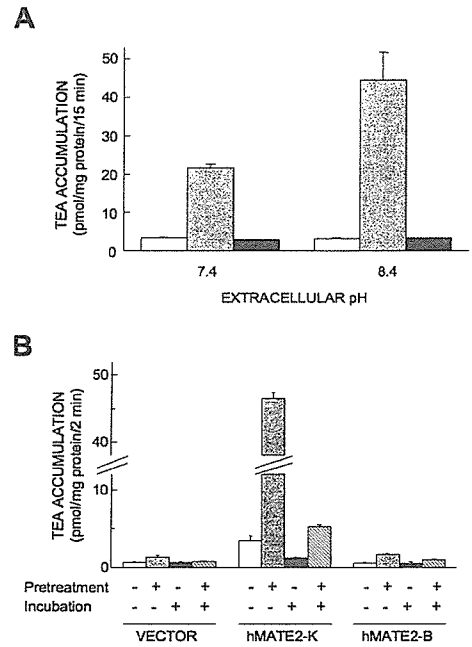

Figure 5. Oppositely directed H^+ gradient–dependent uptake of [^{14}C]tetraethylammonium (TEA) by hMATE2-K but not hMATE2-B in the transiently transfected HEK293 cells. (A) The pcDNA3.1(+) empty vector (□), hMATE2-K cDNA (▨), and hMATE2-B cDNA (■) were transfected into the HEK293 cells. Two days after transfection, uptake of [^{14}C]TEA (5 μM, 7.4 kBq/ml) was examined at the extracellular pH 7.4 or 8.4. (B) HEK293 cells that were transfected with the empty vector, hMATE2-K cDNA, and hMATE2-B cDNA were preincubated with incubation medium (pH 7.4) in the absence (□) or presence (▨) of 30 mM ammonium chloride for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM [^{14}C]TEA (7.4 kBq/ml, pH 7.4) in the absence (■) or presence (▩) of 30 mM ammonium chloride for 2 min at 37°C. Each column represents the mean ± SE of three monolayers.

own activity as an organic cation transporter. We subsequently focused on the functional characterization of hMATE2-K. The hMATE2-K–mediated uptake of [^{14}C]TEA was increased in accordance with the extracellular pH between 6.0 and 9.0 (Figure 7A). Because of the apparent linearity of the time course of the hMATE2-K–mediated uptake of [^{14}C]TEA until 5 min (Figure 7B), the transport characteristics of hMATE2-K at 2 min were selected to examine the kinetics. The linearity of the uptakes of [^{14}C]metformin, [^{3}H]MPP, and [^{3}H]cimetidine also were confirmed until 5 min (data not shown). The uptake of [^{14}C]TEA, [^{14}C]metformin, [^{3}H]MPP, [^{3}H]cimetidine, and [^{14}C]procainamide by hMATE2-K exhibited saturable kinetics, following the Michaelis-Menten equation. The apparent Km values of TEA, metformin, MPP, cimetidine, and procainamide were estimated at 0.83 ± 0.15 mM, 1.05 ± 0.29 mM, 93.5 ± 4.9 μM, 0.37 ± 0.14 mM, and 4.10 ± 0.30 mM, respectively (Figure 8).

Next, we examined the substrate specificity of hMATE2-K. The hMATE2-K mediated the oppositely directed H^+ gradient–dependent transport of structurally diverse organic cations such as [^{14}C]TEA, [^{3}H]MPP, [^{3}H]cimetidine, [^{14}C]metformin, and NMN at extracellular pH 8.4 (Table 2) or at pH 7.4 after

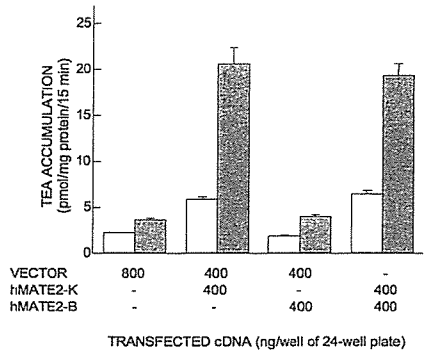

Figure 6. Transfection of hMATE2-B cDNA in HEK293 cells did not stimulate [^{14}C]TEA uptake and did not affect hMATE2-K–mediated [^{14}C]TEA uptake. HEK293 cells that were transfected with the empty vector (800 ng/well), hMATE2-K cDNA (400 ng/well) with the empty vector (400 ng/well), hMATE2-B cDNA (400 ng/well) with the empty vector (400 ng/well), and the combination of both hMATE2-K (400 ng/well) and hMATE2-B (400 ng/well) cDNA were incubated with 5 μM [^{14}C]TEA (7.4 kBq/ml) at pH 7.4 (□) or pH 8.4 (■). Each point represents the mean ± SE of three monolayers.

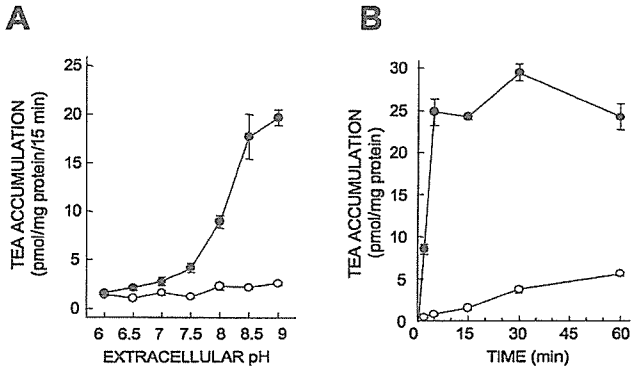

Figure 7. Oppositely directed H^+ gradient dependence (A) and time course (B) of [^{14}C]TEA uptake by hMATE2-K in the transiently transfected HEK293 cells. (A) HEK293 cells that were transfected with the empty vector (○) or hMATE2-K (●) were incubated for 15 min at 37°C with incubation medium of various pH that contained 5 μM of [^{14}C]TEA. Each point represents the mean ± SE of six monolayers by two independent experiments. (B) Time course of [^{14}C]TEA uptake by hMATE2-K. HEK293 cells that were transiently transfected with the empty vector (○) or hMATE2-K cDNA (●) were incubated with [^{14}C]TEA (5μM, 7.4kBq/ml, pH 8.4) for 15 min at 37°C. Each point represents the mean ± SE of three monolayers.

pretreatment of ammonium chloride (Table 3). The uptake of [^{14}C]procainamide was stimulated in hMATE2-K–expressing cells only after pretreatment of ammonium chloride (Tables 2 and 3). In addition, the uptake of [^{14}C]creatinine, [^{3}H]quinidine, [^{3}H]thiamine, and [^{3}H]verapamil was increased in hMATE2-K–expressing cells with treatment of ammonium chloride. However, the anionic compounds *p*-aminohippuric acid, dipeptide

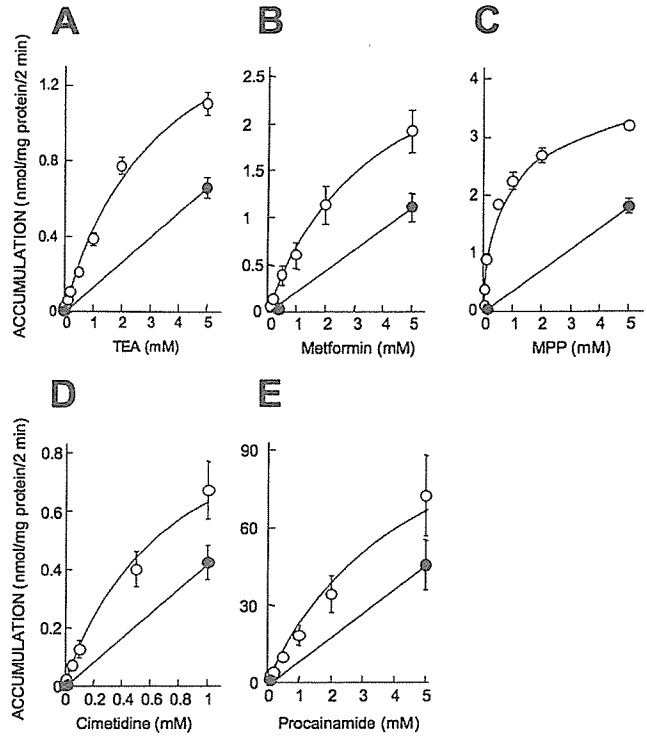

Figure 8. Concentration dependence of hMATE2-K–mediated uptake of [^{14}C]TEA (A), [^{14}C]metformin (B), [^{3}H]1-methyl-4-phenylpyridinium (MPP; C), [^{3}H]cimetidine (D), and [^{14}C]procainamide (E). HEK293 cells that were transfected with hMATE2-K cDNA were incubated with various concentrations of [^{14}C]TEA (pH 8.4; A) or [^{14}C]metformin (pH 8.4; B). For the kinetic analyses of [^{3}H]MPP (C), [^{3}H]cimetidine (D), and [^{14}C]procainamide (E) at pH 7.4, ammonium chloride (30 mM, pH 7.4, 20 min) was pretreated to generate the condition of intracellular acidification. Uptake experiments were performed in the absence (○) or presence of each unlabeled substrate at 10 mM (●) for 2 min at 37°C. Each point represents the mean ± SE of four independent experiments.

glycylsarcosine, and β-lactam antibiotics were not transported by hMATE2-K.

Discussion

In this study, we cloned an alternatively spliced variant of hMATE2 from the human kidney, hMATE2-K (Figures 1 and 2). The hMATE2 that originated from the human brain was not expressed in the kidney (Figure 3). In addition, another variant was cloned from the human brain, hMATE2-B, but original hMATE2 was not cloned from either kidney or brain. hMATE2-B cDNA contains a 154-bp insertion in exon 7 of hMATE2-K and a 46-bp insertion in exon 7 of hMATE2. The hMATE2-B has only 220 amino acids by the 46-bp insertion in exon 7 of MATE2; therefore, the truncated product of the gene, hMATE2-B, seems to lack the transport activity (Figures 5 and 6). Although an RT-PCR band that corresponded to hMATE2-B but not hMATE2 was ubiquitously found, the expression level of the gene was markedly low in consideration of the result of real-time PCR (Figure 3). Consid-

Table 2. Uptake of various organic ions in the HEK293 cells that expressed hMATE2-K at extracellular pH 8.4[a]

Compounds	Vector	hMATE2-K
Uptake (μl/mg protein per 15 min)		
[^{14}C]TEA	0.48 ± 0.00	2.65 ± 0.04[b]
[^{3}H]MPP	2.44 ± 0.61	5.56 ± 0.12[c]
[^{3}H]cimetidine	1.19 ± 0.05	2.08 ± 0.06[b]
[^{14}C]metformin	0.85 ± 0.05	2.48 ± 0.08[b]
[^{14}C]creatinine	2.60 ± 0.05	2.68 ± 0.11
[^{14}C]guanidine	11.0 ± 0.02	8.93 ± 0.21[c]
[^{14}C]procainamide	12.1 ± 0.98	12.0 ± 0.21
[^{14}C]choline	105 ± 0.69	91.5 ± 0.18[b]
[^{3}H]quinidine	137 ± 2.56	148 ± 6.16
[^{3}H]quinine	170 ± 7.83	229 ± 3.99[b]
[^{3}H]thiamine	13.8 ± 0.46	11.5 ± 0.52[c]
[^{3}H]carnitine	10.9 ± 0.67	10.3 ± 0.70
[^{14}C]nicotine	8.16 ± 0.91	9.94 ± 0.18
[^{14}C]captopril	0.45 ± 0.02	0.63 ± 0.05[c]
[^{3}H]verapamil	11.6 ± 0.23	16.0 ± 3.58
[^{14}C]levofloxacin	7.60 ± 1.47	6.66 ± 0.17
[^{3}H]tetracycline	5.86 ± 0.16	6.14 ± 0.01
[^{14}C]*p*-aminohippuric acid	4.90 ± 0.16	4.54 ± 0.13
[^{3}H]glycylsarcosine	0.70 ± 0.07	0.59 ± 0.07
Uptake (μl/mg protein per 10 min)		
NMN	0.47 ± 0.04	1.63 ± 0.65[c]
cephalexin	0.13 ± 0.02	0.14 ± 0.05
cefazolin	0.17 ± 0.03	0.10 ± 0.02
cephradine	0.20 ± 0.03	0.20 ± 0.02

[a]HEK293 cells cultured in a 24-well plate were transfected with the empty vector or hMATE2-K cDNA and incubated for 15 min with incubation medium at pH 8.4 that contained the 19 radiolabeled compounds indicated. After incubation, the radioactivities of solubilized cells were determined. HEK293 cells cultured in a 12-well plate and expressed hMATE2-K were incubated for 10 min with incubation medium at pH 8.4 that contained 1 mM unlabeled N^1-methylnicotinamide (NMN) or β -lactam antibiotics such as cephalexin, cefazolin, and cephradine. After incubation, the amounts of each compound extracted from cells were determined by HPLC. Uptake was expressed as clearance, which was obtained from the net uptake value divided by each substrate concentration in the medium. Data represent the mean ± SEM for three monolayers. MPP, 1-methyl-4-phenylpyridinium acetate; TEA, tetraethylammonium.

[b]$P < 0.01$, [c]$P < 0.05$ significantly different from vector-transfected cells.

ering the 108- and 154-bp deletions in hMATE2-K compared with hMATE2 and hMATE2-B, respectively, the splicing site differed between the kidney and other tissues.

Examination of the tissue distribution clearly indicated that hMATE2-K was a kidney-specific type H^+/organic cation antiporter, whereas hMATE1 mRNA was found in several tissues (Figure 3A). Although hMATE1 mRNA was strongly expressed in the kidney, it also was preferentially expressed in the adrenal gland, liver, skeletal muscle, and testis, corresponding to the report by Otsuka *et al.* (12). Comparing promoter sequences between hMATE1 and hMATE2-K should help to reveal the molecular mechanisms behind the kidney-specific expression of hMATE2-K as well as other renal organic ion transporters hOCT2 (SLC22A2), hOAT1 (SLC22A6), and hOAT3 (SLC22A8) (10). Immunohistochemical examinations clearly demonstrated the apical localization of hMATE2-K protein as well as hMATE1 protein in the proximal tubules (Figure 4, A through F) (12). Considering the functional characteristics and the membrane localization, hMATE2-K was indicated to mediate tubular secretion of cationic compounds at the brush border membranes. These results suggest that some cationic drugs that are preferentially recognized by hMATE2-K are eliminated predominantly through urine *via* tubular secretion, whereas those that are recognized by hMATE1 are excreted into both bile and urine. Therefore, the substrate specificities between these two transporters should be clarified to understand kidney/liver selectivity in the elimination route of the organic cations.

Although the uptake of procainamide was not observed at pH 8.4, it was stimulated by the pretreatment with ammonium chloride (Tables 2 and 3). These results suggest that hMATE2-K requires a strong driving force, a counteracting H^+ gradient across the plasma membrane, for the transport of procainamide. Stoichiometric determination would clarify the coupling ratio between the substrate and H^+ ion or the mass charge of the substrate in combination with the H^+ ion. In addition, further transport studies should be carried out to clarify the precise requirement(s) of the chemical structure(s) to understand the substrate specificity of hMATE2-K.

Table 3. Uptake of various organic ions in HEK293 cells that expressed hMATE2-K at extracellular pH 7.4 after pretreatment of ammonium chloride[a]

Compounds	Vector	hMATE2-K
Uptake (μl/mg protein per 2 min)		
[^{14}C]TEA	0.36 ± 0.04	7.71 ± 0.10[b]
[^{3}H]MPP	3.87 ± 0.07	19.5 ± 0.31[b]
[^{3}H]cimetidine	1.00 ± 0.12	9.24 ± 0.06[b]
[^{14}C]metformin	0.56 ± 0.01	6.68 ± 0.42[b]
[^{14}C]creatinine	0.58 ± 0.05	0.93 ± 0.07[c]
[^{14}C]guanidine	3.10 ± 0.18	3.71 ± 0.05[c]
[^{14}C]procainamide	14.9 ± 0.11	18.5 ± 0.67[b]
[^{14}C]choline	7.64 ± 0.14	8.08 ± 0.21
[^{3}H]quinidine	108 ± 2.01	126 ± 2.21[b]
[^{3}H]quinine	109 ± 0.60	124 ± 5.10[c]
[^{3}H]thiamine	5.98 ± 0.06	12.4 ± 5.10[b]
[^{3}H]carnitine	12.16 ± 0.08	12.7 ± 0.26
[^{14}C]nicotine	7.05 ± 0.32	7.97 ± 0.20
[^{14}C]captopril	0.32 ± 0.01	0.27 ± 0.02
[^{3}H]verapamil	24.09 ± 1.54	30.5 ± 0.05[c]
[^{14}C]levofloxacin	9.29 ± 0.19	10.1 ± 0.83
[^{3}H]tetracycline	3.46 ± 0.16	4.06 ± 0.19
[^{14}C]*p*-aminohippuric acid	1.14 ± 0.03	1.33 ± 0.12
[^{3}H]glycylsarcosine	0.26 ± 0.01	0.33 ± 0.03
Uptake (μl/mg protein per 10 min)		
NMN	0.51 ± 0.03	2.54 ± 0.36[b]
cephalexin	0.13 ± 0.01	0.16 ± 0.03
cefazolin	0.14 ± 0.05	0.07 ± 0.01
cephradine	0.27 ± 0.04	0.30 ± 0.03

[a]HEK293 cells cultured in a 24-well plate for the uptake examination of radiolabeled compounds or cultured in a 12-well plate for unlabeled compounds were transfected with the empty vector or hMATE2-K cDNA and incubated for 20 min with incubation medium that contained 30 mM ammonium chloride. After washing, 2- or 10-min uptake measurements at extracellular pH 7.4 were carried out for radiolabeled or unlabeled compounds as described in the legend for Table 2, respectively. Data represent the mean ± SEM for three monolayers.
[b]$P < 0.01$, [c]$P < 0.05$ significantly different from vector-transfected cells.

In this study, an antihyperglycemic agent, metformin, was demonstrated for the first time to be a good substrate for hMATE2-K (Figure 8B, Tables 2 and 3). There is no information available about the H^+ gradient–dependent transport of metformin in renal brush border membrane vesicles. Actually, approximately 70% of metformin in the circulation was eliminated in urine mainly *via* tubular secretion, and when co-administered, cimetidine decreased the renal clearance of metformin (23,24). Recently, we demonstrated that metformin is a superior substrate for renal OCT2 rather than hepatic OCT1 (17,25). Renal distribution of metformin immediately after intravenous administration was almost 23-fold higher than the plasma concentration in the rats (1.29 μg/ml in plasma *versus* 29.8 μg/g in kidney at 3 min after intravenous administration) (17). This background and our results strongly suggest that renal hMATE2-K plays a key role in the tubular secretion of metformin after basolateral accumulation by renal hOCT2.

Conclusion

An active variant, hMATE2-K, and a longer variant, hMATE2-B, were cloned and characterized. It is indicated that hMATE2-K is the first kidney-specific H^+/organic cation antiporter to mediate the tubular secretion of a wide range of cationic compounds across the brush border membranes in the proximal tubules. The physiologic roles of hMATE2-B and hMATE2, including the expressional characteristics of these genes, still are unclear. Further studies should be performed to elucidate the physiologic and pharmacologic significance of hMATE2-K as well as hMATE1 in the renal handling of ionic drugs.

Acknowledgments

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Short Communication

Molecular Cloning, Functional Characterization and Tissue Distribution of Rat H^+/Organic Cation Antiporter MATE1

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Purpose. Transport characteristics and tissue distribution of the rat H^+/organic cation antiporter MATE1 (multidrug and toxin extrusion 1) were examined.
Methods. Rat MATE1 cDNA was isolated by polymerase chain reaction (PCR) cloning. Transport characteristics of rat MATE1 were assessed by HEK293 cells transiently expressing rat MATE1. The mRNA expression of rat MATE1 was examined by Northern blot and real-time PCR analyses.
Results. The uptake of a prototypical organic cation tetraethylammonium (TEA) by MATE1-expressing cells was concentration-dependent, and showed the greatest value at pH 8.4 and the lowest at pH 6.0–6.5. Intracellular acidification induced by ammonium chloride resulted in a marked stimulation of TEA uptake. MATE1 transported not only organic cations such as cimetidine and metformin but also the zwitterionic compound cephalexin. MATE1 mRNA was expressed abundantly in the kidney and placenta, slightly in the spleen, but not expressed in the liver. Real-time PCR analysis of microdissected nephron segments showed that MATE1 was primarily expressed in the proximal convoluted and straight tubules.
Conclusions. These findings indicate that MATE1 is expressed in the renal proximal tubules and can mediate the transport of various organic cations and cephalexin using an oppositely directed H^+ gradient.

KEY WORDS: H^+/organic cation antiporter; kidney; MATE1; renal secretion.

INTRODUCTION

The secretion of drugs and xenobiotics is an important physiological function of the renal proximal tubules. Functional studies using isolated membrane vesicles and cultured renal epithelial cells have suggested that the renal tubular secretion of cationic substances involves concerted actions of two distinct classes of organic cation transporters: one facilitated by the transmembrane potential difference in the basolateral membrane and the other driven by the transmembrane H^+ gradient (H^+/organic cation antiporter) in the brush-border membrane (1,2). The membrane-potential dependent organic cation transporters (OCT1-3, SLC22A1-3) have been identified and well characterized (3–5), but the molecular nature of the H^+/organic cation antiporter has not been elucidated for a long time.

Recently, based on in silico homology screening, human and mouse orthologues of the multidrug and toxin extrusion (MATE) family, which confers multidrug resistance to bacteria, have been identified (6). The tissue distribution, membrane localization and transport characteristics of human MATE1 suggested that this transporter is similar to transporters of the renal H^+-coupled organic cation export system (6). However, the precise substrate specificity based on direct uptake measurements and expression profiles of wide-ranging tissues for MATE1 have not been evaluated. So far, we have characterized the functional properties of H^+/organic cation antiporters in the rat kidney (7–12). In the present study, we isolated rat MATE1 cDNA and investigated the transport characteristics and tissue and intrarenal distribution of this transporter.

MATERIALS AND METHODS

Materials

Cephalexin (Shionogi, Osaka, Japan) and cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan) were donated by the respective suppliers. [^{14}C]Tetraethylammonium bromide (TEA) (2.035 GBq/mmol), [^{14}C]creatinine (2.035 GBq/mmol) and [^{14}C]procainamide (2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals Inc (St. Louis, MO). [^{14}C]Metformin (962 MBq/mmol) and [^{14}C]guanidine hydrochloride (1.961 Gbq/mmol) were purchased from Moravek Biochemicals Inc (Brea, CA). [^{3}H]1-Methyl-4-phenylpyridinium acetate (MPP) (2.7 TBq/mmol) and [^{14}C]*p*-aminohippurate (PAH) (1.9 GBq/mmol) were from PerkinElmer Life Analytical Sciences (Boston, MA). [*N*-Methyl-^{3}H]Cimetidine (451 GBq/mmol) was obtained from

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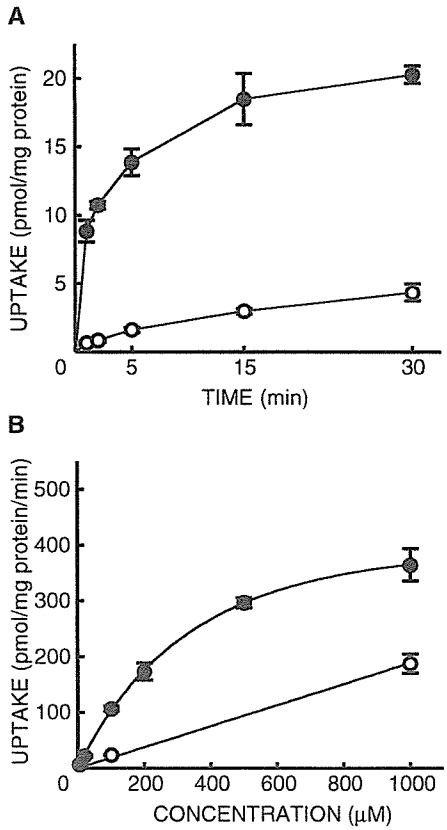

Fig. 1. Transport of [^{14}C]TEA by rat MATE1. **A** Time course of [^{14}C]TEA uptake by HEK293 cells transiently expressing rat MATE1. HEK293 cells transfected with vector alone (pcDNA3.1) (○) or MATE1 cDNA (●) were incubated with 5 μM of [^{14}C]TEA (pH 8.4) at 37°C. Each point represents the mean ± S.E. for three monolayers. **B** Concentration-dependence of [^{14}C]TEA uptake by HEK293 cells transiently expressing rat MATE1. HEK293 cells transfected with rat MATE1 cDNA were incubated with various concentrations of [^{14}C]TEA (pH 8.4) in the absence (●) or presence of 5 mM TEA (○) for 1 min at 37°C. This figure shows representative data of three separate experiments. Each point represents the mean ± S.E. for three monolayers.

Amersham Biosciences (Uppsala, Sweden). N^1-Methylnicotinamide (NMN) was purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available.

cDNA Cloning of Rat MATE1

Rat MATE1 cDNA was isolated from Marathon-Ready rat kidney cDNA (Clontech, Palo Alto, CA) using specific primers designed based on sequence information of the NCBI reference sequence NM_001014118 and human MATE1 (6). The rat MATE1 cDNA was cloned using the following primers: forward 5′-CACATGGAGGTCTTG GAGGAGCCTGCGCCG-3′ and reverse 5′-CACAGACT GAGGAGCACCTGCATTGCTGG-3′. The PCR product was subcloned into the expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA), and sequenced by a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan). The nucleotide sequence for the open reading frame of rat MATE1 was identical to the NCBI reference sequence NM_001014118 except for G1608T, which substituted Tyr for Asp at position 529 (variant 2). This transporter had the same ability to transport [^{14}C]TEA and [^{14}C]metformin as the reference type of MATE1 (variant 1). The nucleotide sequences reported here have been submitted to the DDBJ/EMBL/GenBank Data Bank with Accession No. AB248823 (variant 1) and No. AB248824 (variant 2).

Cell Culture and Transfection

HEK293 cells (American Type Culture Collection CRL-1573) were cultured as described previously (13–15). pcDNA 3.1 (+) containing cDNA encoding rat MATE1 was transfected into HEK293 cells using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. At 48 h after the transfection, the cells were used for uptake experiments.

Uptake Experiments in HEK293 Cells

The cellular uptake of various radiolabeled compounds was measured by monolayers grown on poly-D-lysine-coated 24-well plates as described previously (13–15). Typically, the cells were preincubated with 0.2 ml of incubation medium (pH 7.4) for 10 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium (pH 8.4) containing a radiolabeled compound such as [^{14}C]TEA was added. After an appropriate period of incubation, the medium was aspirated, and the monolayers were washed twice with 1 ml of ice-cold incubation medium. The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. Furthermore, the cellular uptake of cephalosporin antibiotics was measured as described previously (15). For cellular uptake of NMN, HEK293 cells expressing rat MATE1 were incubated with NMN for 1 h, washed two times, and scraped with 0.5 ml of incubation medium (pH 7.4). The accumulation of NMN was determined according to the method of Musfeld *et al.* (16). The conditions for high-performance liquid chromatography (HPLC) were as follows: column, Zorbax ODS column 4.6 mm inside diameter × 250 mm (Du Pont, Wilmington, DE, USA); mobile phase, 5 mM sodium heptanesulfonate containing 0.5% triethylamine (pH 3.2):acetonitrile = 78:22; flow rate, 1.0 ml/min; excitation and emission wavelengths, 366 and 418 nm, respectively; temperature, 40°C.

Northern Blot Analysis and Real-time PCR for Various Tissues and Microdissected Nephron Segments

The preparation of total RNA and Northern blot analysis under the high-stringency conditions were performed as described previously (17). Total RNA from various rat tissues was purchased from BioChain (Hayward, CA), and reverse transcribed (18). Isolation of total RNA from microdissected nephron segments and reverse transcription (RT) were previously reported (19,20). Using these RT products, real-time PCR was carried out (18). The primer-probe set used for rat MATE1 was as follows: forward primer, 5′-GGG CATCGCTGCTAACCTT-3′ (bp 567–585); reverse primer, 5′-CCCCAAGATGTAGCTGATGGA-3′ (bp 654–634); fluorescence probe, 5′-(6-Fam) TCAACGCCCTGGCCAA

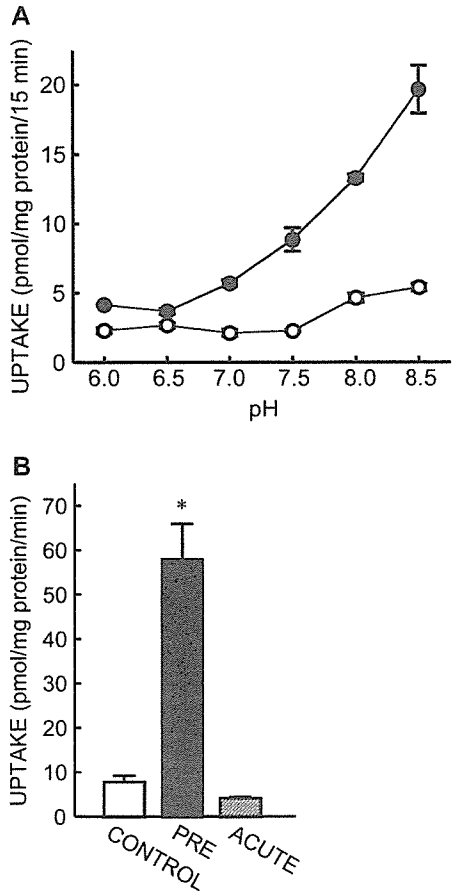

Fig. 2. Effect of pH on the transport of [^{14}C]TEA by rat MATE1. **A** Effect of extracellular pH on [^{14}C]TEA uptake by HEK293 cells transiently expressing rat MATE1. HEK293 cells transfected with vector alone (pcDNA3.1) (○) or rat MATE1 cDNA (●) were incubated with 5 μM of [^{14}C]TEA (pH 8.4) for 15 min at 37°C. **B** Effect of intracellular pH on [^{14}C]TEA uptake by HEK293 cells transiently expressing rat MATE1. HEK293 cells transfected with MATE1 cDNA were preincubated with incubation medium (pH 7.4) in the absence (CONTROL and ACUTE) or presence (PRE) of 30 mM ammonium chloride for 20 min (21). Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [^{14}C]TEA (pH 8.4) in the absence (CONTROL and PRE) or presence (ACUTE) of 30 mM ammonium chloride for 1 min at 37°C. Each point represents the mean ± S.E. for three monolayers. *$P < 0.05$, significantly different from CONTROL.

CTATCTGTTT (Tamra)(phosphate)-3′ (bp 587–612). The primer-probe sets used for rat OCT1 and OCT2 were predeveloped TaqMan Assay Reagents (Applied Biosystems, Foster, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also measured as an internal control with GAPDH Control Reagent (Applied Biosystems).

Statistical Analyses

Data were analyzed statistically using a non-paired *t* test (Fig. 3B) or a one-way analysis of variance followed by Sheffé's test (Fig. 2B). *P* values of less than 0.05 were considered significant. In all figures, when error bars are not shown, they are smaller than the symbol.

RESULTS

We cloned rat MATE1 cDNA from rat kidney by PCR cloning. Rat MATE1 showed 79% amino acid identity with human MATE1, 91% with mouse MATE1, 48% with human MATE2 and 57% with mouse MATE2 (6). When rat MATE1 was expressed in HEK293 cells, a time- and concentration-dependent uptake of [^{14}C]TEA was observed (Fig. 1). The uptake by MATE1 exhibited saturable kinetics, following the Michaelis–Menten equation. The apparent Km value of the uptake was calculated at 570 ± 64 μM.

Figure 2 shows the effects of changes in extra- and intracellular pH on [^{14}C]TEA uptake by MATE1. When the extracellular pH was changed from 6.0 to 8.4, the uptake was greatest at pH 8.4 and lowest at pH 6.0–6.5. Intracellular pH

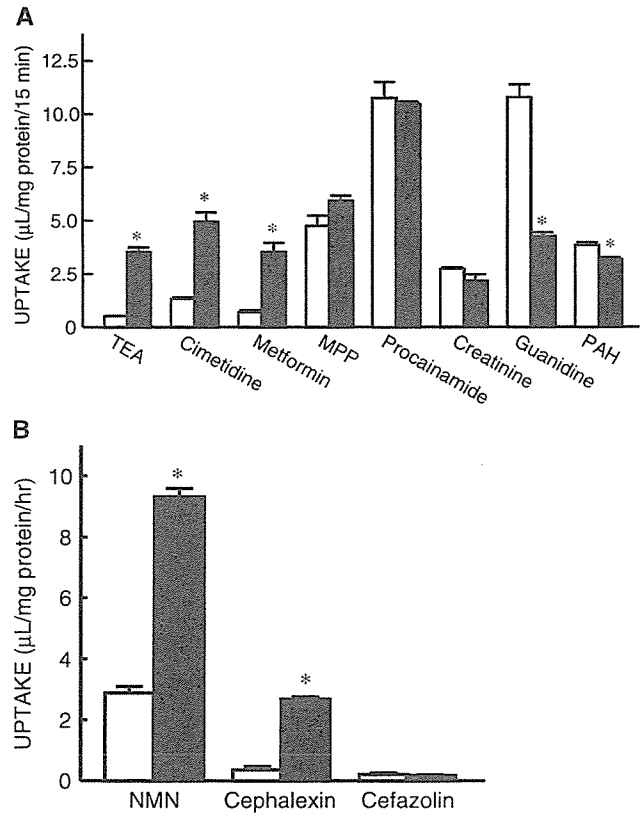

Fig. 3. Uptake of various compounds by rat MATE1. **A** Uptake of various radiolabeled compounds by HEK293 cells transiently expressing rat MATE1. HEK293 cells transfected with vector alone (pcDNA3.1) (*open column*) or MATE1 cDNA (*closed column*) were incubated with [^{14}C]TEA (5 μM), [^{3}H]cimetidine (23 nM), [^{3}H]MPP (3.8 nM), [^{14}C]metformin (10 μM), [^{14}C]creatinine (5 μM), [^{14}C]guanidine (5 μM), [^{14}C]procainamide (5 μM) or [^{14}C]PAH (5 μM) for 15 min at 37°C. After the incubation, the radioactivity of solubilized cells was determined. **B** Uptake of NMN, cephalexin and cefazolin by HEK293 cells transiently expressing rat MATE1. HEK293 cells transfected with vector alone (pcDNA3.1) (*open column*) or MATE1 cDNA (*closed column*) were incubated with each compound (1 mM) for 1 h at 37°C. After the incubation, amounts of each compound extracted from cells were determined by HPLC. Uptake was expressed as clearance, which was obtained by dividing the net uptake value by the concentration of each substrate in the medium. Each column represents the mean ± S.E. for three monolayers.

was manipulated by treating the cells with ammonium chloride according to a previous report (21). When ammonium chloride is added to a preincubation medium and then removed (pretreatment), the intracellular pH falls. However, the exposure of cells to ammonium chloride (acute treatment) causes a rapid alkalization of the intracellular pH. As shown in Fig. 2B, intracellular acidification through pretreatment resulted in a marked stimulation of [^{14}C]TEA uptake, whereas the uptake was reduced by acute treatment.

We then examined the substrate specificity of MATE1. As shown in Fig. 3, MATE1 mediated the transport of several organic cations with different chemical structures such as [^{14}C]TEA, [^{3}H]cimetidine, [^{14}C]metformin, and NMN. In addition, MATE1 transported the zwitterionic cephalosporin cephalexin, which was demonstrated to be a substrate for the rat H^+/organic cation antiporter in the membrane vesicle studies (8). In contrast, MATE1 did not transport cationic compounds such as [^{3}H]MPP, [^{14}C]procainamide, [^{14}C]creatinine and [^{14}C]guanidine, the anionic compound [^{14}C]PAH, or the anionic cephalosporin cefazolin.

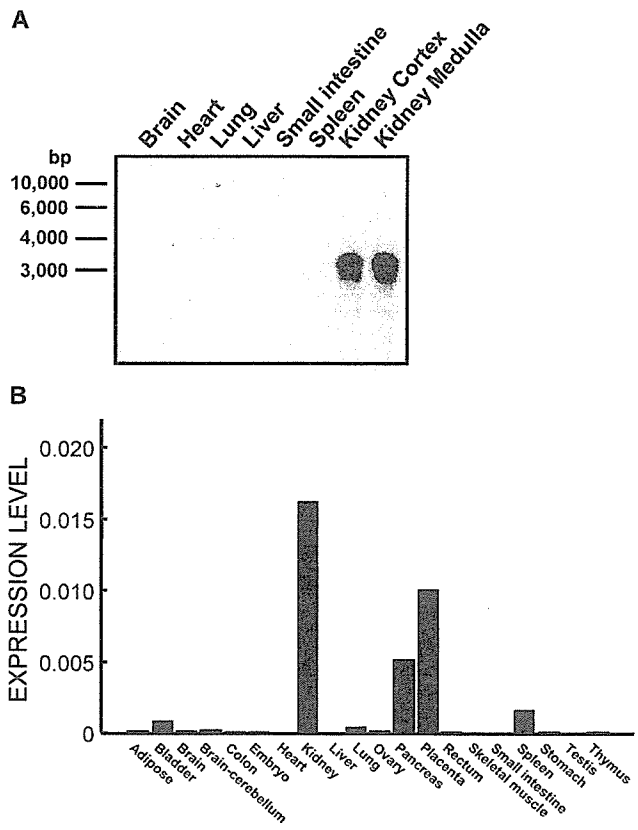

Fig. 4. Tissue distribution of rat MATE1. **A** Northern blot analysis of MATE1 mRNA in rat tissues. Total RNA (30 μg) from the indicated tissues was electrophoresed, blotted, and hybridized with a specific probe for rat MATE1 under high stringency conditions. **B** Real-time PCR for MATE1 mRNA in rat tissues. RNA from various rat tissues was reverse-transcribed, and rat MATE1 and GAPDH mRNA levels were determined by real-time PCR using an ABI PRISM 7700 sequence detector. The mRNA expression level of rat MATE1 is represented as a ratio of that of GAPDH in each tissue. Each column represents the mean for two separate experiments.

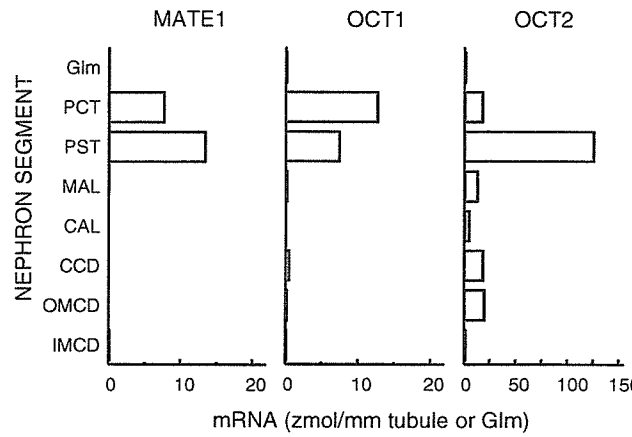

Fig. 5. Distribution of rat MATE1, OCT1 and OCT2 mRNAs along the microdissected renal nephron segments. Each PCR was performed using part of RT reaction derived from 10 glomeruli and 5 mm of renal tubules. The mRNA expression levels of rat MATE1, OCT1 and OCT2 were determined by real-time PCR using an ABI PRISM 7700 sequence detector. Each column represents the mean for two separate experiments. *Glm* glomerulus, *PCT* proximal convoluted tubule, *PST* proximal straight tubule, *MAL* medullary thick ascending limb, *CAL* cortical thick ascending limb, *CCD* cortical collecting duct, *OMCD* outer medullary collecting duct, *IMCD* inner medullary collecting duct.

Finally, tissue distribution of rat MATE1 mRNA was examined. High-stringency Northern blot analysis revealed that a transcript (about 3.5 KB) of rat MATE1 was strongly expressed in the kidney cortex and medulla, and faintly in the spleen (Fig. 4A). No positive signal was detected in other tissues, including the brain, small intestine and liver (Fig. 4A). The results of real-time PCR analyses using RNA from various rat tissues were consistent with the results of the Northern blot analysis. MATE1 mRNA was highly expressed in the kidney and placenta, and slightly expressed in the pancreas, spleen, bladder and lung (Fig. 4B).

To determine the distribution of rat MATE1 mRNA along the nephron segments, real-time PCR analysis using microdissected nephron segments was performed, as comparing with those of rat OCT1 and OCT2 mRNA. As shown in Fig. 5, rat MATE1 mRNA was primarily expressed in the proximal convoluted tubule (PCT) and proximal straight tubule (PST). The expression of rat OCT1 mRNA was also found in the PCT and PST. Rat OCT2 mRNA was highly expressed in PST with about 10 fold higher expression level as compared with that of rat MATE1 or OCT1 mRNA, and significant expression was also detected in other segments such as PCT, outer medullary collecting duct (OMCD) and cortical collecting duct (CCD). Distribution of rat OCT1 and OCT2 mRNA was corresponded to a previous report (20).

DISCUSSION

Using renal brush-border membrane vesicles, the transport of TEA (7,22,23), aminocephalosporins (8), cimetidine (9,24,25), NMN (26) and procainamide (27) was examined, and demonstrated to be actively driven by an outwardly directed H^+ gradient via the H^+/organic cation antiport

system. By using a renal epithelial cell line LLC-PK_1, the apical transport of TEA (28,29) and procainamide (30) was demonstrated to be mediated by the H^+/organic cation antiport system. In the present study, we found that most of these organic compounds were transported by rat MATE1. However, procainamide was not transported, although rat OCT1 and OCT2 can transport procainamide (unpublished data). As the transport of procainamide via the H^+/organic cation antiport system was demonstrated in rabbit renal brush-border membrane vesicles (27) and porcine LLC-PK_1 cells (30), a species difference may be responsible for this discrepancy. Using human renal brush-border membrane vesicles, Chun *et al.* (31) found that transport of guanidine was stimulated by H^+ gradient, and that the mechanism involved was distinct from that for the transport of TEA and NMN. Although species differences should be taken into consideration, the present findings that guanidine was not transported by rat MATE1 may support the membrane vesicle study (31). Taken together, these findings suggest that other MATE isoforms reported in humans (6) may be involved in the transport of procainamide and guanidine in the renal brush-border membrane. Further studies are needed to clarify the molecular mechanisms responsible for the diversity of organic cation transport in the renal brush-border membrane.

Otsuka *et al.* (6) reported that [^{14}C]TEA transport by human MATE1 was inhibited by MPP, but not by NMN. In the present study, rat MATE1 was able to transport NMN, but not MPP. These findings suggest that human and rat MATE1 have different properties of substrate recognition for these compounds. Experimental conditions such as inhibition studies (6) and our uptake studies may also explain the different findings.

Rat MATE1 mRNA is highly expressed in the kidney, especially in the proximal tubules, and placenta, but not in the liver. In contrast, human MATE1 is preferentially expressed in the kidney and liver, but not in the placenta (6), indicating a clear species difference in the distribution of MATE1 mRNA between human and rat. Previous studies using vesicles prepared from rat sinusoidal membranes (32) and from human term placenta (33) revealed the presence of a H^+/organic cation antiport system in these tissues. Detailed functional characterization suggested that the system in the liver and placenta differs from the renal system (32,33), and MATE1 mRNA expression in rat and human tissues supports these assumptions. Other molecules than MATE1 may be responsible for the H^+/organic cation antiport in the rat liver and human placenta.

The transport of organic cations such as TEA and cimetidine in the renal brush-border membrane was mediated by H^+/organic cation antiport system energized by a transmembrane H^+ gradient (1,2). Since the luminal pH is more acidic than the intracellular pH in the proximal tubules (34), due to an Na^+/H^+ antiporter and/or ATP-driven H^+-pump, it is reasonable to assume that the inward H^+ gradient (luminal pH<intracellular pH) can drive the secretion of organic cations *in vivo*. Human MATE1 exhibited pH-dependent transport of TEA in cellular uptake and efflux studies (6), but these analyses are not enough to prove the H^+/TEA antiport mechanism. It is possible that pH-dependent transport of TEA may be due to the modulation of transport activity caused by changes in extracellular pH. In the present study, we found that intracellular acidification by ammonium chloride pretreatment resulted in the marked stimulation of [^{14}C]TEA uptake by MATE1, suggesting that outwardly directed H^+ gradient serves as a driving force for MATE1 (Fig. 2B).

In conclusion, rat MATE1 is abundantly expressed in the renal proximal tubules, and can accept various compounds including organic cations and zwitterionic cephalosporin as substrates. Changes in extra- and intracellular pH suggested that an oppositely directed H^+ gradient works as a driving force for MATE1. Although further studies are needed to elucidate the physiological and pharmacokinetic roles of this transporter, the present findings can provide important information about the renal tubular secretion of organic cations.

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Human Organic Anion Transporter 3 Gene Is Regulated Constitutively and Inducibly via a cAMP-Response Element

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ABSTRACT

Human organic anion transporter (OAT) 3 (SLC22A8) is localized to the basolateral membranes of renal tubular epithelial cells and plays a critical role in the excretion of anionic compounds. We previously reported that interindividual variation in the OAT3 mRNA level corresponded to interindividual differences in the rate of renal excretion of cefazolin. However, there is little information available on the molecular mechanisms regulating the gene expression of OAT3. Therefore, in the present study, we examined the transcriptional regulation of human OAT3. A deletion analysis of the OAT3 promoter suggested that the region spanning −214 to −77 base pairs was essential for basal transcriptional activity. This region contained a perfectly conserved cAMP-response element (CRE), and a mutation here led to a reduction in promoter activity. Electrophoretic mobility shift assays showed that CRE-binding protein (CREB)-1 and activating transcription factor (ATF)-1 bound to CRE. The activity of the OAT3 promoter was increased through the phosphorylation of CREB-1 and ATF-1 by treatment with 8-bromo-cAMP. This paper reports the first characterization of the human OAT3 promoter and shows that CREB-1 and ATF-1 function as constitutive and inducible transcriptional regulators of the human OAT3 gene via CRE.

The kidney plays important roles in the excretion of various drugs, toxins, and endogenous metabolites. The excretion process consists of three steps: glomerular filtration, tubular secretion, and reabsorption. Transporters expressed in renal tubular epithelial cells are mainly involved in the tubular secretion and reabsorption (Pritchard and Miller, 1996; Inui and Okuda, 1998; Wright and Dantzler, 2004). The organic anion transporter (OAT) family (OAT1–4), which is predominantly expressed in the kidney (Russel et al., 2002; Sekine et al., 2006), mediates the transport of many anionic compounds, such as β-lactam antibiotics, nonsteroidal anti-inflammatory drugs, antiviral drugs, and antitumor drug, and it regulates their excretion (Inui et al., 2000; Burckhardt and Burckhardt, 2003; Sweet, 2005).

We previously found that the mRNA level of OAT3 was higher than that of any other member of the organic ion transporter (SLC22A) family in the human kidney, and OAT3 was localized to the basolateral membranes of proximal tubules (Motohashi et al., 2002). OAT3 possessed greater activity to transport cephalosporin antibiotics, including cefazolin than OAT1 in vitro experiment (Ueo et al., 2005). Furthermore, clinical pharmacokinetic and gene expression analyses showed that only the mRNA level of OAT3 among OAT1–4 significantly correlated with the apparent elimination rate constant of the free fraction of cefazolin in patients with mesangial proliferative glomerulonephritis ($r = 0.757$; $p < 0.01$) (Sakurai et al., 2004, 2005).

The OAT3 mRNA level is assumed to be mainly under the control of transcriptional regulation. However, little is known about the functional characteristics of the promoter region of OAT3. Based on this background, we cloned the human OAT3 promoter region and examined its promoter activity using opossum kidney (OK) cells. This is the first report to identify the *cis*-element and *trans*-factors for the regulation of the human OAT family in the kidney.

Materials and Methods

Materials. [γ-^{32}P]ATP was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Restriction enzymes were from New England Biolabs (Beverly, MA). Antibodies used for supershift

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ABBREVIATIONS: OAT, organic anion transporter; OK, opossum kidney; 8-Br-cAMP, 8-bromo-cAMP; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; PKA, protein kinase A; EMSA, electrophoretic mobility shift assay; CRE, cAMP-response element; mut, mutation/mutated; ATF, activating transcription factor; CREB, CRE-binding protein; SNP, single-nucleotide polymorphism; cSNP, coding single-nucleotide polymorphism; rSNP, regulatory single-nucleotide polymorphism.

assays were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Those used for Western blotting were purchased from Cell Signaling Technology Inc. (Beverly, MA). 8-Bromo-cAMP sodium salt (8-Br-cAMP) was obtained from Sigma-Aldrich (St. Louis, MO).

5′-Rapid Amplification of cDNA Ends. To identify the transcription start site of human OAT3, 5′-rapid amplification of cDNA ends (RACE) was carried out using Human Kidney Marathon-Ready cDNA (Clontech, Mountain View, CA) according to the manufacturer's instructions. The primers for 5′-RACE were as follows: a gene-specific primer for OAT3 (accession no. NM_004254), 5′-CCCACTCTGTCACAATGGAGTCCTTGG-3′ (473 to 446); and a nested gene-specific primer for OAT3, 5′-CCCATGCTTCCCACACG-GTCCAGGATC-3′ (177 to 151). The PCR products were subcloned into the pGEM-T Easy Vector (Promega, Madison, WI) and sequenced using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

Cloning of the 5′-Regulatory Region of the OAT3 Gene. Based on the human genomic sequence (accession no. NT_033903), the 2488-base pair flanking region upstream of the transcription start site was cloned by PCR using the primers listed in Table 1 and human genomic DNA (Promega). The PCR product was isolated by electrophoresis and subcloned into the firefly luciferase reporter vector pGL3-Basic (Promega), at NheI and XhoI sites. This full-length reporter plasmid is hereafter referred to as −2488/+21.

Preparation of Deletion Reporter Constructs. The −1862/+21 construct was generated by digestion of the −2488/+21 construct with SacI, and the 5′-deleted constructs (−926/+21, −214/+21) were generated by digestion of the −2488/+21 construct with MluI and either SpeI or ApaI. The ends were blunted with T4 DNA polymerase (Takara Bio, Otsu, Japan) and then self-ligated. The −77/+21 and −11/+21 constructs were generated by PCR with primers containing an NheI site and XhoI site (Table 1). The site-directed mutations in the putative CCAAT box and CRE were introduced into the −214/+21 construct with a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers listed in Table 1. The nucleotide sequences of these deleted or mutated constructs were verified.

Cell Culture, Transfection, and Luciferase Assay. OK cells were cultured in medium 199 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) without antibiotics, in an atmosphere of 5% CO_2, 95% air at 37°C and subcultured every 7 days using 0.02% EDTA and 0.05% trypsin. OK cells were plated into 24-well plates (4×10^6 cells/well) and transfected the following day with the reporter constructs and 25 ng of the *Renilla reniformis* vector pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. The firefly and *Renilla* activities were determined 48 h after the transfection using a dual-luciferase assay kit (Promega) and a LB940 luminometer (Berthold, Bad Wildbad, Germany). The firefly activity was normalized to *Renilla* activity. For the protein kinase A (PKA) stimulation experiment, the cells were treated with 1 mM 8-Br-cAMP for 12 h before the luciferase assay.

Electrophoretic Mobility Shift Assay. Nuclear extract was prepared from OK cells according to the method of Shimakura et al. (2005). The double-stranded oligonucleotides used in the EMSA are listed in Table 1. The OAT3 probe (−101/−75) was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara Bio), and the labeled probe was purified through a Sephadex G-25 column (GE Healthcare). EMSA was performed according to Alimov et al. (2003) but with some modifications. The OK nuclear extract (10 μg) was incubated in binding buffer [120 mM KCl, 20 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol, 5% glycerol, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 10 mM NaF, 100 μM Na_3VO_4, and 2% protease inhibitor cocktail] for 30 min at 4°C. Thereafter, the labeled probe was added, and the mixture was incubated for a further 30 min at 4°C. For competition experiments and supershift assays, excess (50-fold) unlabeled oligonucleotide and antibodies (1 μg) were added 30 min before the addition of the labeled probe, respectively. The volume of the binding mixture was 20 μl throughout the experiment. The DNA-protein complex was then separated on a 4% polyacrylamide gel for 1.5 h at 200 V and room temperature in 0.5× Tris borate-EDTA buffer. Gels were dried and exposed to X-ray film for autoradiography.

Western Blot Analysis. OK cells were treated with phosphate-buffered saline in the presence or absence of 1 mM 8-Br-cAMP for 15 min before the preparation of nuclear extracts. Twenty micrograms of nuclear extract was separated on a 10% SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). Membrane blocking and antibody incubations were carried out using the PhosphoPlus CREB (Ser133) Antibody kit (Cell Signaling Technology Inc.) according to the manufacturer's instructions. The bound antibody was detected on X-ray film by enhanced chemiluminescence with horse-

TABLE 1
Oligonucleotide sequence of primers
NheI and XhoI sites are underlined. Mutations introduced into the oligonucleotides are shown in boldface.

Name	Sequence (5′-3′)	Position
Primers for cloning of the OAT3 promoter		
OAT3/−2488NheI-F	GG<u>GCTAGC</u>GTGATAGATCCCCCAATAAGG	−2488 to −2468
OAT3/+21XhoI-R	GG<u>CTCGAG</u>GCAGCTCAGCTCTAACAAGC	+2 to −21
Primers for the −77/+21 and −11/+21 deletion constructs		
OAT3/−77NheI-F	CT<u>GCTAGC</u>CCGCAAAAGAAAGTCAAACAT	−77 to −57
OAT3−11NheI-F	CT<u>GCTAGC</u>GGCACAAACACAGCTTGTTAG	−11 to +10
OAT3/+21XhoI-R	GG<u>CTCGAG</u>GCAGCTCAGCTCTAACAAGC	+2 to −21
Primers for the site-directed mutagenesis		
mCCAAT-F	CACAGCACTCTCCCTG**TCT**GTGACGTTAATCCGC	−107 to −74
mCCAAT-R	GCGGATTAACGTCAC**AGA**CAGGGAGAGTGCTGTG	−74 to −107
mCRE-F	CACAGCACTCTCCCTGCCAGTGA**TA**TTAATCCGCAAAAG	−107 to −69
mCRE-R	CTTTTGCGGATTAA**TA**TCACTGGCAGGGAGAGTGCTGTG	−69 to −107
Oligonucleotides for EMSA		
OAT3 (−101−75)-F	ACTCTCCCTGCCAGTGACGTTAATCCG	−101 to −75
OAT3 (−101−75)-R	CGGATTAACGTCACTGGCAGGGAGAGT	−75 to −101
mutCCAAT-F	ACTCTCCCTG**TTT**GTGACGTTAATCCG	−101 to −75
mutCCAAT-R	CGGATTAACGTCAC**AAA**CAGGGAGAGT	−75 to −101
mutCRE-F	ACTCTCCCTGCCAGTGA**GCC**TAATCCG	−101 to −75
mutCRE-R	CGGATTA**GGC**TCACTGGCAGGGAGAGT	−75 to −101
Double mut-F	ACTCTCCCTG**TTT**GTGA**GCC**TAATCCG	−101 to −75
Double mut-R	CGGATTA**GGC**TCAC**AAA**CAGGGAGAGT	−75 to −101
Sp1 consensus-F	ATTCGATCGGGGCGGGGCGAGC	
Sp1 consensus-R	GCTCGCCCCGCCCCGATCGAAT	

radish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare).

Data Analysis. The results were expressed relative to pGL3-Basic and represent the mean ± S.D. of three replicates. Two or three experiments were conducted, and representative results are shown. In the mutational and PKA stimulation experiments, the statistical analysis was performed with the one-way analysis of variance followed by Scheffé F-post hoc testing.

Results

Determination of the Transcription Start Site of OAT3. Sequencing of the longest RACE product showed that the terminal position of OAT3 cDNA was located 126 nucleotides above the start codon, which was 10 base pairs downstream of the 5′ end of OAT3 cDNA registered in the National Center for Biotechnology Information database (accession no. NM_004254). Therefore, the 5′ end of OAT3 cDNA was numbered with +1 as the transcription start site in this study.

Determination of Minimal OAT3 Promoter. To determine the minimal region required for basal activity of the promoter, a series of deletion constructs were transfected into OK cells, and luciferase activity was measured (Fig. 1A).

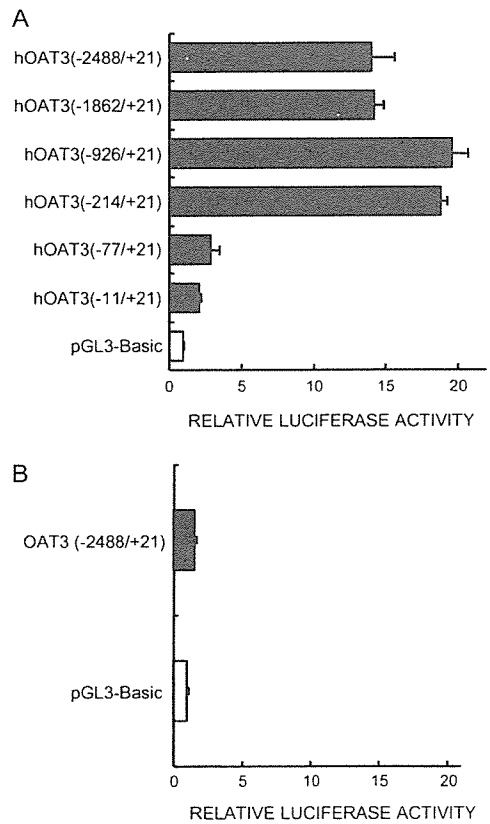

Fig. 1. Identification of the transcriptional activity of the human OAT3 promoter in OK cells. A, deletion analysis of the human OAT3 promoter in OK cells. A series of deleted promoter constructs [equimolar amounts of the −2488/+21 construct (500 ng)] were transfected into OK cells for luciferase assays. B, transcriptional activity of the human OAT3 promoter in human embryonic kidney 293 cells. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative -fold increase compared with pGL3-Basic and represent the mean ± S.D. of three replicates.

OK cells were used in the luciferase assay because they have an organic anion transport system (Hori et al., 1993), and the transcription factors and/or cofactors required for the expression exist intrinsically in these cells. The longest reporter constructs (−2488/+21) showed an approximately 14-fold increase in luciferase activity compared with pGL3-Basic in OK cells, but they had little promoter activity in human embryonic kidney 293 cells, which lack an organic anion transport system (Fig. 1B). The 5′-deleted constructs (−1862/+21, −926/+21, −214/+21) had the same level of activity as the longest construct, −2488/+21. In contrast, the −77/+21 construct had one-sixth of the activity of −214/+21. These results suggested that the elements important for the basal promoter activity were located between −214 and −77.

Figure 2 shows the results of a computational analysis of the −214/−77 region of the OAT3 promoter, using TRANSFAC 6.0 at www.gene-regulation.com/. This analysis revealed that there is one putative CCAAT box and one perfectly conserved cAMP-response element (CRE) in this region, suggesting that these sites contribute to the transcriptional regulation of OAT3.

Mutagenesis of CCAAT Box and CRE. To determine whether these sites were important for the promoter activity of OAT3, mutations at these sites (designated as mCCAAT and mCRE, respectively) were introduced in the −214/+21 construct and transfected into OK cells. As shown in Fig. 3, mCCAAT and mCRE reduced the luciferase activity to two-third and one-third of the wild-type level, respectively. These results suggest that the CCAAT box and CRE are responsible for the basal promoter activity of OAT3.

Electrophoretic Mobility Shift Assay. To confirm which transcription factors bind to these elements, EMSA was performed using an OAT3 probe (−101/−75) containing both the CCAAT box and CRE and nuclear extract from OK cells. The probe (−101/−75) formed a DNA-protein complex (Fig. 4A, lane 2). The formation of the complex was prevented by the addition of an excess amount of unlabeled oligonucleotide (−101/−75) but not by unrelated oligonucleotide (Fig. 4A, lanes 3 and 7), suggesting that transcription factors bind to the probe (−101/−75). Next, we prepared three oligonucleotides, mutCCAAT lacking a CCAAT box, mutCRE lacking a CRE, and double mut lacking both elements to determine which sites the protein recognized. mutCCAAT impaired the formation of the complex, but mutCRE and double mut did not (Fig. 4A, lanes 4–6), suggesting that the

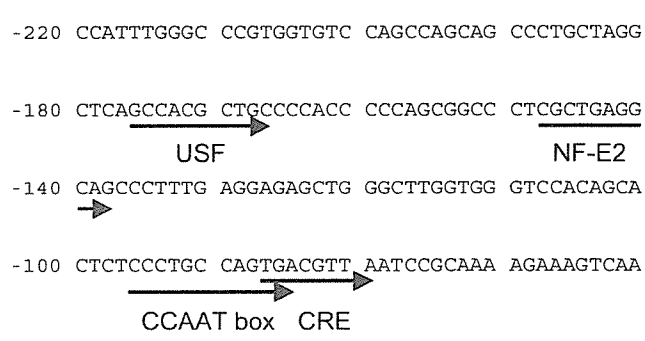

Fig. 2. Nucleotides sequence of the promoter region (−220 to −1) of human OAT3. Numbering is relative to the transcription start site. The putative binding sites for the transcription factors are indicated (the arrows indicate the direction).

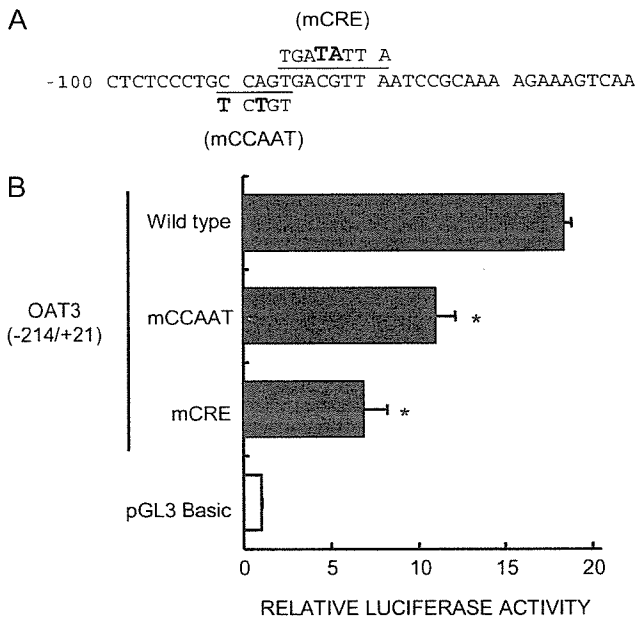

Fig. 3. Mutational analysis of the putative CCAAT box and CRE of the human OAT3 promoter. A, schematic of the mutated OAT3 (−214/+21) reporter constructs. B, these constructs (500 ng) were transfected into OK cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative -fold increase compared with pGL3-Basic and represent the mean ± S.D. of three replicates. *, significantly different from wild type; $p < 0.05$.

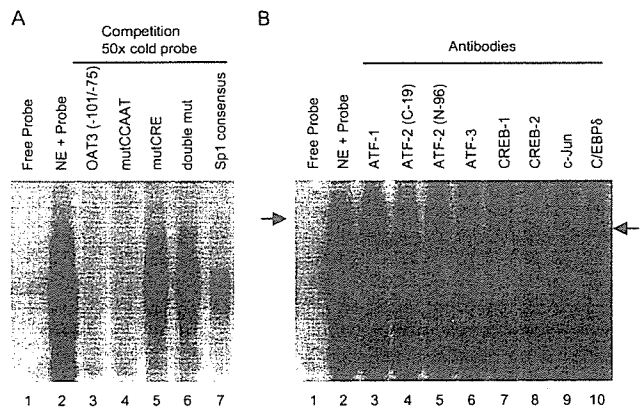

Fig. 4. EMSA using nuclear extract from OK cells and a human OAT3 probe (−101/−75). A, nuclear extract from OK cells was incubated with the ^{32}P-labeled OAT3 oligonucleotide probe (−101/−75) alone (lane 2) or in the presence of excess unlabeled oligonucleotide (−101/−75) (lane 3), excess mutated oligonucleotide (lane 4–6), and excess Sp1 oligonucleotide (lane 7). In lane 1, nuclear extract was not added. B, nuclear extract from OK cells was incubated with the ^{32}P-labeled OAT3 oligonucleotide probe (−101/−75) alone (lane 2) or in the presence of antibody against ATF-1 (lane 3), ATF-2 (C-19) (lane 4), ATF-2 (*N*-96) (lane 5), ATF-3 (lane 6), CREB-1 (lane 7), CREB-2 (lane 8), c-Jun (lane 9), and CCAAT/enhancer-binding protein δ (lane 10). In lane 1, nuclear extract was not added. Arrows indicate the supershifted complexes.

proteins that bind to the probe (−101/−75) recognize CRE, but not the CCAAT box.

It has been demonstrated that various transcription factors bind to CRE (Hai and Hartman, 2001). Figure 4B shows results of supershift assays using antibodies against transcription factors that bind to CRE. Antibodies against activating transcription factor (ATF)-1 and CRE-binding protein (CREB)-1 were able to supershift the DNA-protein complex (Fig. 4B, lanes 3 and 7). In contrast, antibodies against ATF-2, ATF-3, CREB-2 (ATF-4), c-Jun, and CCAAT/enhancer-binding protein δ did not result in a supershift (Fig. 4B, lanes 4–6 and 8–10). These results indicate that the transcription factor, which binds to CRE, consists entirely of a homodimer or heterodimer made up of ATF-1 and/or CREB-1.

Effect of PKA Activation. Both ATF-1 and CREB-1 are phosphorylated by PKA and activate the transcription of target genes. The effect of the PKA activator 8-Br-cAMP on the activity of the OAT3 promoter was investigated with the −214/+21, mCRE, and −77/+21 constructs in OK cells. Treatment with 8-Br-cAMP increased luciferase activity 2.5-fold in the −214/+21 construct. In contrast, the response to 8-Br-cAMP was diminished in the mCRE and −77/+21 constructs (Fig. 5). As shown in Fig. 6, levels of phosphorylated CREB-1 and ATF-1 increased with 8-Br-cAMP treatment. These results suggested that PKA stimulated the OAT3 promoter through phosphorylation of both CREB-1 and ATF-1.

Discussion

In the present study, we performed a functional promoter assay of human OAT3 and found that CRE is responsible for the basal and inducible promoter activity of OAT3 and that CREB-1 and ATF-1 bind to CRE. CREB-1 was first described as a transcription factor mediating induction by extracellular signals activating adenylate cyclase and PKA (Gonzalez and Montminy, 1989). Thereafter, it was demonstrated that this transcription factor is responsible for the constitutive transcriptional regulation without stimuli (Quinn, 1993). ATF-1 is capable of dimerizing with CREB-1 (Hurst et al., 1991), but it cannot stimulate gene expression unless it is combined with PKA (Rehfuss et al., 1991). It is, therefore, suggested that CREB-1 is involved in the constitutive expression of OAT3. This is the first paper to identify the *cis*-element and *trans*-factor for the regulation of the human OAT family in the kidney.

Soodvilai et al. (2004) demonstrated that the transport

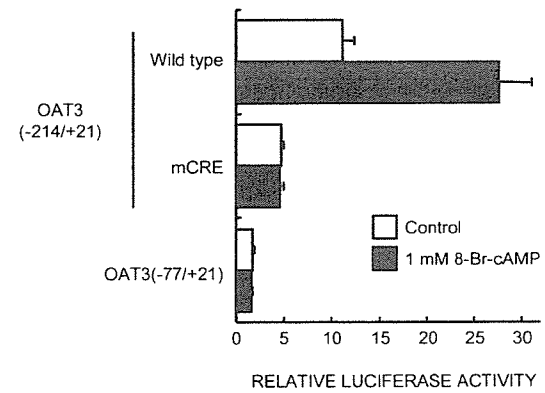

Fig. 5. Effect of 8-Br-cAMP on the promoter activity of various reporter constructs. These constructs (500 ng) were transfected into OK cells for luciferase assays. 8-Br-cAMP (1 mM) was added to the medium 36 h after transfection, and luciferase assays were carried out after 12 h. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Open columns and closed columns indicate control and 8-Br-cAMP treatment, respectively. Data are reported as the relative -fold increase compared with pGL3-Basic and represent the mean ± S.D. of three replicates. *, significantly different from control; $p < 0.05$.

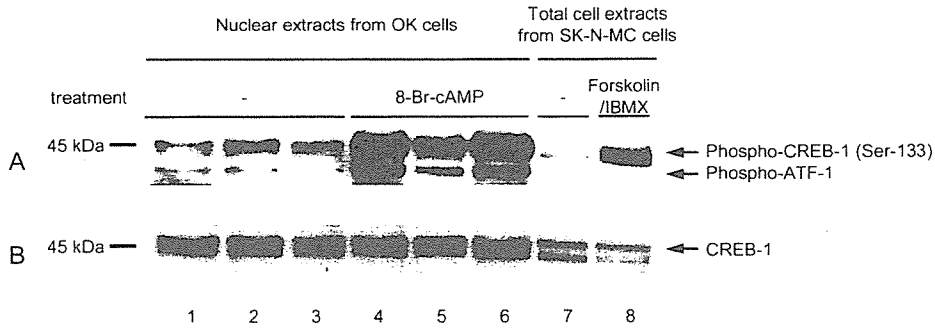

Fig. 6. Western blot analyses of nuclear extracts from OK cells treated without (lanes 1–3) or with 1 mM 8-Br-cAMP for 15 min (lanes 4–6), and total cell extracts from SK-N-MC cells, prepared without or with forskolin/3-isobutyl-1-methylxanthine (IBMX) treatment, to serve as negative and positive controls. Nuclear extracts or total cell extract (20 μg) was separated on a 10% SDS polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. Phospho-CREB (Ser133) antibody (A) and CREB antibody (B) (1:1000 dilution) were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies. The arrowheads indicate the positions of each transcription factor.

activity of rabbit OAT3 is up-regulated by PKA (10-min stimulation) and speculated that this effect is due to the transfer of additional OAT3 transporters from an intracellular compartment to the basolateral cell membrane. Conversely, we showed that phosphorylation of CREB-1 and ATF-1 by PKA through the stimulation of 8-Br-cAMP (12 h) increased the promoter activity of OAT3. The present study revealed another possible molecular mechanism for the activation of PKA to stimulate the transport activity of OAT3, although it is unclear whether the rabbit OAT3 promoter has a CRE. It is therefore suggested that the short-term as well as long-term regulation of OAT3 is mediated by the activation of PKA. Further studies are needed to clarify the physiological and pharmacological implications of PKA signaling for the transport activity of OAT3.

In this study, a mutation in the CCAAT box reduced the luciferase activity, although no proteins bound to the CCAAT box in the EMSA experiments. Such a mutation may affect the function of CREB-1 and reduce the luciferase activity because the CCAAT box is in the vicinity of the CRE. It was reported that mutations that disrupted sequences located 5′ and 3′ of the CRE (TTACGTCA) in the promoter of the phosphoenolpyruvate carboxylkinase gene caused less severe reductions in basal promoter activity (Quinn et al., 1988), suggesting that not only the CRE but also the regions around the CRE are important for the constitutive transcriptional regulation of OAT3.

To clarify the interindividual variation in the pharmacokinetics of drugs, single-nucleotide polymorphisms (SNPs) in the coding region (cSNP) of drug transporters have been investigated (Ishikawa et al., 2004). Erdman et al. (2006) examined the allele frequency of several cSNPs in the OAT3 gene and transport characteristics and found that allele frequencies of cSNPs, which resulted in a completed loss of function, were very low (Xu et al., 2005; Erdman et al., 2006). In addition, the cSNP of OAT3 was considered to be unlikely to influence the pharmacokinetics of drugs (Nishizato et al., 2003; Sakurai et al., 2005). Recent studies have demonstrated that SNP in the promoter region (regulatory SNP; rSNP) is a candidate for the cause of the variation in the pharmacokinetics among individuals. Analyses of rSNP in the *multidrug resistance 1* gene suggested that several SNPs comprise a haplotype, influencing multidrug resistance 1 mRNA expression (Taniguchi et al., 2003; Takane et al., 2004). Screening of the rSNP of OAT3 is needed to identify

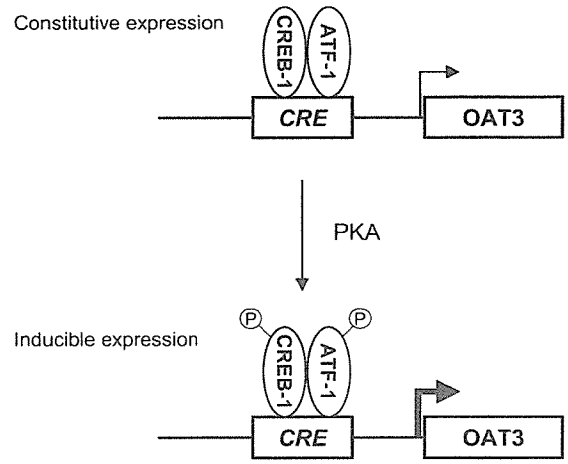

Fig. 7. Schematic model of transcriptional regulation of the human OAT3 gene. CREB-1 and ATF-1 bind to CRE and activate the transcription of the OAT3 gene (constitutive expression). PKA further activates the transcription of the OAT3 gene through phosphorylation of both CREB-1 and ATF-1 (inducible expression).

the genomic information affecting the mRNA level of OAT3. Alternatively, the regulation or modulation of CREB-1 may be involved in the interindividual difference in OAT3 mRNA levels.

In conclusion, the present study indicates that CRE is involved in the constitutive and inducible transcriptional regulation of the human OAT3 gene (Fig. 7). This is the first report to identify the *cis*-element for the regulation of the OAT family that is predominantly expressed in the kidney. It is possible that the regulation and modulation of CREB-1 affect OAT3 mRNA levels. To elucidate the interindividual variation in OAT3 mRNA expression, these factors should be taken into consideration.

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Short Communication

Interactions of Fluoroquinolone Antibacterials, DX-619 and Levofloxacin, with Creatinine Transport by Renal Organic Cation Transporter hOCT2

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Interactions of DX-619, a novel fluoroquinolone antibacterial, and levofloxacin (LVFX) with the human renal organic cation transporter hOCT2 were studied. The intracellular accumulation of [^{14}C]creatinine in stable transfectants of HEK293 cells expressing hOCT2 (hOCT2-HEK293) as well as vector-transfected HEK293 cells (VEC-HEK293) was evaluated in the presence of DX-619 and LVFX at various concentrations. When added extracellularly, both DX-619 and LVFX inhibited the uptake of [^{14}C]creatinine (5 μM) by hOCT2-HEK293 cells in a dose-dependent manner. Unlike in hOCT2-HEK293 cells, the uptake in VEC-HEK293 cells was not inhibited by either fluoroquinolone suggesting that hOCT2 was specifically involved in the inhibition. The apparent IC_{50} value for the inhibition of [^{14}C]creatinine uptake in hOCT2-HEK293 cells was 1.29 ± 0.23 μM for DX-619 and 127 ± 27 μM for LVFX, indicating DX-619 to be ~100-fold more potent than LVFX at inhibiting the transport of [^{14}C]creatinine by hOCT2. A Dixon plot revealed that the inhibition by DX-619 of the hOCT2-mediated transport of [^{14}C]creatinine was competitive. Fluoroquinolone antibacterials have the ability to inhibit the transport of creatinine by hOCT2, with DX-619 being much more effective than LVFX.

Key words: DX-619; levofloxacin; hOCT2; creatinine; fluoroquinolone; organic cation transporter

Introduction

The level of creatinine in serum is the most commonly used clinical index of renal function. It is well recognized that the serum creatinine level is influenced by factors such as the patient's age and sex as well as the method of its determination. Because creatinine is mostly eliminated through urine *via* glomerular filtration, its renal clearance is regarded to be proportional to renal function. However, significant secretion of creatinine occurs at renal tubules, and may cause an overestimation of the glomerular filtration rate (GFR) especially in patients with decreased renal function.[1-3] Shemesh *et al.*[3] reported that the tubular secretion of creatinine is relatively constant regardless of a decreased glomerular filtration rate. Attempting to obtain a better estimation of the glomerular filtration rate by using creatinine clearance, Berglund *et al.*,[4] Burgess *et al.*[5] and van Acker *et al.*[6] administered trimethoprim[4] and cimetidine[5,6] to patients in which the tubular secretion of creatinine was blocked. Similarly, the application of cationic drugs to patients could lead to incorrect estimations of renal function because of decreased tubular secretion of creatinine.

In the renal proximal tubules, organic ion transporters mediate the tubular secretion of ionic drugs, thus contributing to the efficient extrusion of harmful substances from the body.[7-11] Human organic cation transporter 2, hOCT2, is the most abundant organic cation transporter so far reported in the human kidney.[12] hOCT2 should play significant roles in the basolateral translocation of some H_2-receptor antagonists[13,14] and biguanides[15] into epithelial cells in the renal proximal tubules. hOCT2 also accepts endogenous monoamines such as norepinephrine, serotonin, histamine and dopamine.[16] We found that creatinine is a specific substrate for the organic cation transporter hOCT2 expressed at the basolateral membranes of the human

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Abbreviations used are: hOCT2, human organic cation transporter 2; hOCT1, human organic cation transporter 1; GFR, glomerular filtration rate; LVFX, levofloxacin; MPP, 1-methyl-4-phenylpyridinium.