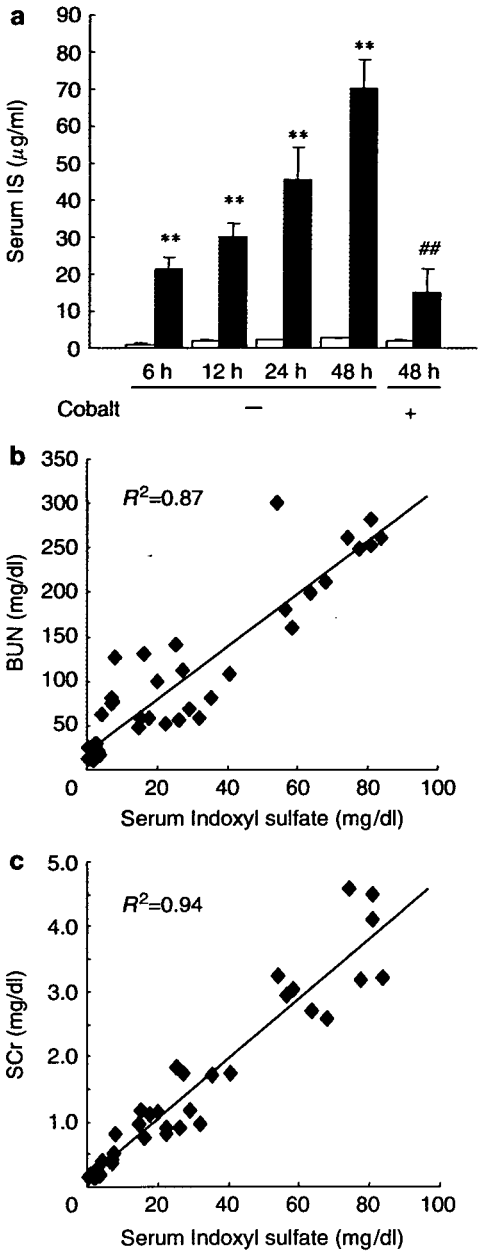

Figure 3 | Serum IS concentration in control and ischemic rats with and without cobalt intake. (**a**) High-performance liquid chromatography analysis of serum to determine the concentration of IS after sham operation (open column) or I/R injury (closed column). Each column represents the mean ± s.e.m. for three to eight rats. $^{**}P<0.01$, significantly different from control rats at the same period. $^{\#\#}P<0.01$, significantly different from rats without cobalt intake over the same period. Correlation between (**b**) BUN or (**c**) SCr and serum IS concentration.

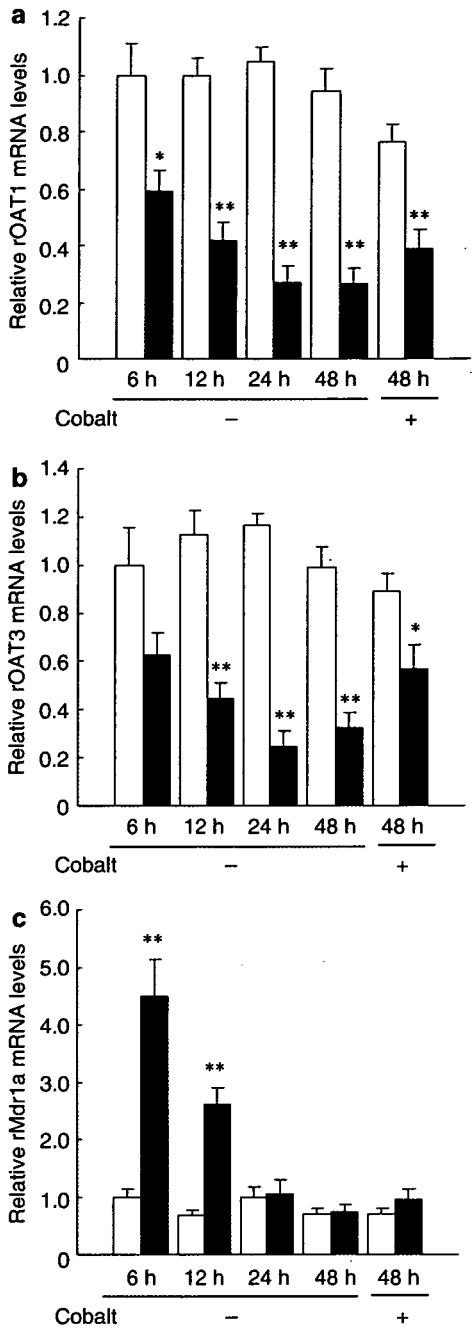

Figure 4 | mRNA expression of organic anion transporters and rMdr1a in ischemic rat kidney. Relative mRNA expression levels of (**a**) rOAT1, (**b**) rOAT3 and (**c**) rMdr1a in control (open column) and ischemic rats (closed column) with and without cobalt intake. The expression level in the control at 6 h was set at 1.0. Each column represents the mean ± s.e.m. for three to eight rats. $^{*}P<0.05$, $^{**}P<0.01$, significantly different from control rats at the same period.

efficiently mediate ES transport with high affinity in addition to transporting several anionic compounds such as PAH, methotrexate, prostaglandin E_2, and cyclic adenosine mono phosphate.[1] Therefore, downregulation of both rOAT1 and rOAT3 should account for the decrease in PAH and ES uptake at the basolateral membrane of tubular cells in ischemic rat kidney. Intriguingly, we also observed a significant recovery in the accumulation of ES, but not PAH, by renal slices derived from I/R-induced rats given water supplemented with cobalt. *In vitro* studies have shown that the contribution of rOAT1 and rOAT3 to the uptake of IS is 38 and 62%, respectively.[32] Therefore, the decrease in serum IS levels in ischemic rats treated

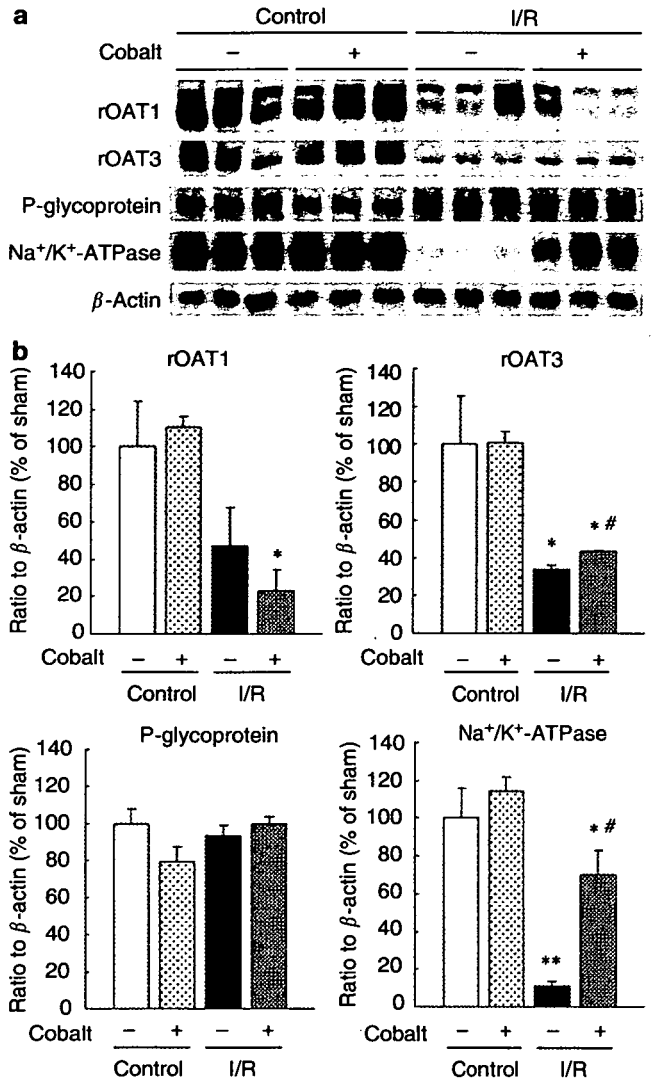

Figure 5 | Immunoblots of kidneys from control and ischemic rats with or without cobalt intake 48 h after ischemia. (**a**) Antiserum specific for rOAT1, rOAT3, P-glycoprotein, Na^+/K^+-ATPase, and β-actin were used as primary antibodies. (**b**) The ratio of rOAT1, rOAT3, P-glycoprotein, and Na^+/K^+-ATPase density to β-actin density. The values for control rats without cobalt intake were arbitrarily defined as 100%. Each bar represents the mean ± s.e.m. for three rats in each group. $^*P<0.05$, $^{**}P<0.01$, significantly different from control rats with or without cobalt intake; $^{\#}P<0.05$, significantly different from ischemic rats without cobalt intake.

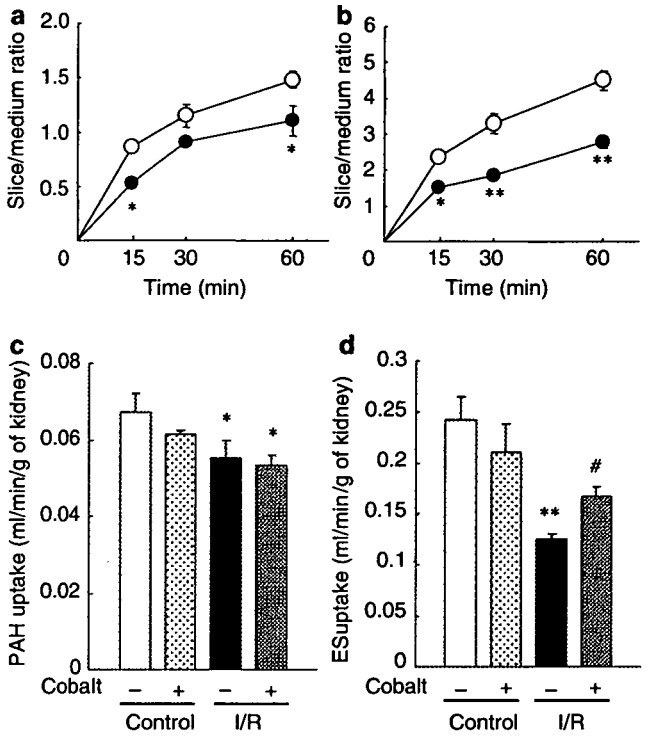

Figure 6 | Renal uptake of PAH and ES in control and ischemic rats with or without cobalt intake. (**a**) PAH and (**b**) ES accumulation by renal slices of control and ischemic rats at 48 h after ischemia. Renal slices from control (○) and ischemic rats (●) were incubated at 37°C in incubation buffer containing 5 μM [^{14}C]PAH or 5 μM [^{3}H]ES, for the period indicated. D-[^{3}H]Mannitol or [^{14}C]mannitol was used to estimate the extracellular trapping and nonspecific uptake of [^{14}C]PAH or [^{3}H]ES, respectively. Each point represents the mean ± s.e.m. for three to five slices. $^*P<0.05$, $^{**}P<0.01$, significantly different from control rats at same period. The renal uptake clearance of (**c**) PAH and (**d**) ES in control and ischemic rats with or without cobalt intake at 48 h after ischemia. Renal slices from rats were incubated at 37°C in incubation buffer containing 5 μM [^{14}C]PAH or 5 μM [^{3}H]ES, for 60 min. $^*P<0.05$, $^{**}P<0.01$, significantly different from control rats with or without cobalt intake; $^{\#}P<0.05$, significantly different from ischemic rats without cobalt intake.

cobalt might be the result of partially restored rOAT3 function and/or expression.

Alternatively, the driving force of rOAT1 and rOAT3 activity could be depressed in renal tubular cells of I/R kidney. rOAT1 and rOAT3 are known to mediate exchange of anionic substrates with dicarboxylic acids, including α-ketoglutarate, where the concentration in the cytoplasm is much higher than in the serum, producing an outward-directed driving force for influx of anionic substrates.[33,34] Na^+/K^+-ATPase actively pumps Na^+ out of the renal proximal tubule cells sustaining an inwardly directed Na^+ gradient.[35] The inward movement of Na^+ drives the uptake of α-ketoglutarate by Na^+/dicarboxylate cotransporter 3.[35] The depression of Na^+/K^+-ATPase expression in the ischemic rat kidney eliminates the inward Na^+ gradient, thereby decreasing uptake of α-ketoglutarate by Na^+/dicarboxylate cotransporter 3. The protein level of Na^+/K^+-ATPase in the ischemic rat kidney was significantly restored to the expression level found in the control rat kidney by ingestion of cobalt. In ischemic renal tubules, the driving force may not be fully maintained, thereby resulting in reduced uptake of IS *via* rOAT3. The preventive effect of cobalt intake on the level of serum IS could involve restoration of the driving force for the rOAT1 and rOAT3 transporters. Cobalt intake might block the production of factors that decrease the driving force for these transporters. Alternatively, the activity of the efflux transporter(s) for IS at the apical membrane of renal tubules, which is reduced by I/R, might be restored by ingestion of cobalt. The precise mechanism involved in the relationship between the levels of

serum IS and the expression levels of OATs and the I/R-induced downregulation of these transporters should be further studied.

It was reported that the level of P-glycoprotein increased in rat kidney after glycerol-induced ARF.[36] In this study, we found that renal rMdr1 mRNA showed a transient upregulation during I/R of the rat kidney. To our knowledge, this is the first report indicating upregulation of rMdr1 in the kidney after I/R injury. Mdr1 is one of the known genes transcriptionally regulated by hypoxia-inducible factor 1 under hypoxic conditions.[17] Therefore, it is suggested that hypoxia-inducible factor 1 induced by ischemia stimulates the transient induction of rMdr1 gene. However, there was no significant difference in the P-glycoprotein level between the control and the ischemic rats at 48 h after ischemia, as found in the rMdr1 mRNA expression. P-glycoprotein may be upregulated during the early stage of ischemia, thereby preventing tubular cell derangement by secreting cytotoxic substances produced in ischemic kidney.

In conclusion, we report that I/R of rat kidney induces the serum accumulation of IS, which could be caused by the downregulation of renal rOAT1 and rOAT3, accompanied by decreased transport activities. Although ingestion of cobalt ameliorates the progression of renal dysfunction, as evident from the decrease in the level of BUN, SCr, and IS, it has no significant effect on the expression of rOAT1 and rOAT3. ES uptake in renal slices of ischemic rats suggests that ingestion of cobalt causes a partial restoration of transport activity of rOAT3, but not rOAT1. This study has increased our understanding the mechanism of regulation and pathophysiological implications of OATs of renal tubules as well as the renal handling of anionic drugs and endogenous toxins under ischemic ARF conditions.

MATERIALS AND METHODS

Materials

Cobalt chloride was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). [6,7-^{3}H(N)]-ES ammonium salt (2120 GBq/mmol), D-[1-^{3}H(N)]-mannitol (525.4 GBq/mmol), *p*-[Glycyl-^{14}C]-aminohippuric acid (1.95 GBq/mmol) and D-[1-^{14}C]-mannitol (1.89 GBq/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). IS was obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were of the highest purity available.

Experimental animals

Male Sprague–Dawley rats, initially weighing 150–180 g (Crea Japan Inc., Tokyo, Japan), were housed in a standard animal maintenance facility at constant temperature (21–23°C), humidity (50–70%), and a 12:12 h light/dark cycle for at least 1 week before the day of the experiment. All animal experiments were conducted according to the guidelines of Kumamoto University for the care and use of laboratory animals. Rats in the control group were given tap water, whereas rats in the cobalt-intake group were given water containing 2 mM cobalt chloride 24 h before the induction of ischemic injury to kill.

Rats were anesthetized using sodium pentobarbital (50 mg/kg intraperitoneally), and placed on a heating plate (39°C) to maintain a constant temperature. The kidneys were exposed via midline abdominal incisions. Renal ischemia was induced by vascular clamps (AS ONE, Osaka, Japan) over both pedicles for 30 min. After the clamps were released, the incision was closed in two layers with 3-0 sutures. Sham animals (control) underwent anesthesia, laparotomy, and renal pedicle dissection only. All animals received warm saline solution instilled in the peritoneal cavity during the surgical procedure, and were then allowed to recover with *ad libitum* access to food and water. Animals were killed under surgical anesthesia at 6, 12, 24, and 48 h after experimental intervention. Blood samples were collected for measurement of SCr, BUN, sodium (Na), chloride (Cl), and potassium (K) in serum. A 50 μl aliquot of serum was added directly to 100 μl of methanol. After centrifugation at $3000 \times g$ for 10 min, the supernatant was assayed by high-performance liquid chromatography. For histological assessment, kidney samples were fixed in 4% buffered formaldehyde. Paraffin sections of the excised kidney were stained with hematoxylin–eosin reagent. For semiquantitative analysis of morphologic change, 10 high-magnification fields (0.0675 mm^2) of outer stripe and inner stripe of outer medulla in rats were randomly selected. Then, tubular injury was graded with an arbitrary score of 0–3: 0, the absence of necrosis; 1, mild; 2, moderate; and 3, severe. All quantification was performed in a blinded manner.

High-performance liquid chromatography determination of IS

The high-performance liquid chromatography system consisted of a Shimadzu LC-10ADVP pump and a Shimadzu RF-10AXL fluorescence spectrophotometer. A column of LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as the stationary phase and the mobile phase consisted of acetate buffer (0.2 M, pH 4.5). The flow rate was 1.0 ml/min. IS was detected by means of a fluorescence monitor (excitation 280 nm, emission 375 nm).

mRNA isolation and cDNA synthesis

For mRNA extraction, MagNA Pure LC mRNA Isolation Kit II (Roche Diagnostics, Basel, Switzerland) was used according to the instruction manual. In this automated process, the tissue samples were dissolved in a buffer containing a chaotropic salt and an RNase inactivator. The samples were homogenized with Lysing Matrix D (Qbiogene Inc., Morgan Irvine, CA, USA) and FastPrep (Qbiogene Inc.) at an oscillation speed of 4.5 for 30 s. The 3′-poly (A^+) from the released mRNA hybridizes to the added biotin-labeled oligo(dT). This complex is immobilized onto the surface of streptavidin-coated magnetic beads. After a DNase digestion step, unbound substances were removed by several washing steps, and purified mRNA was eluted (elution volume, 50 μl) with a low-salt buffer. cDNA was synthesized by using High-Capacity cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA). Briefly, 50 μl of mRNA substrate, 10 μl of reverse transcription (RT) buffer, 4 μl of dNTP mixture, 10 μl of RT random primers, 5 μl of MultiScribe reverse transcriptase, and 21 μl of nuclease-free water were used for cDNA synthesis. After each reverse transcription, cDNA was stored at −30°C.

Real-time polymerase chain reaction

We performed a TaqMan quantitative real-time RT polymerase chain reaction using ABI PRISM 7900 sequence to determine the expression level of rOAT1, rOAT3, rMdr1a and eukaryotic

18S ribosomal RNA (18 S rRNA). TaqMan 18S rRNA control reagents, the primer sets and products of TaqMan Gene Expression Assays were purchased from Applied Biosystems Inc. as follows: rOAT1, Rn00568143_m1; rOAT3, Rn00580082_m1; rMdr1a, Rn00591394_m1; and 18S rRNA, 4319413E.

Western blot analysis

Kidneys were homogenized in a homogenization buffer comprising 230 mM sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethanesulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A. After measurement of the protein content using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA), each sample was mixed in a loading buffer (2% sodium dodecyl sulfate, 125 mM Tris-HCl, 20% glycerol, 5% 2-mercaptoethanol) and heated at 100°C for 2 min. The sample were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) by semi-dry electroblotting. The blots were blocked overnight at 4°C with 2% ECL advance Blocking agents (GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA) in Tris-buffered saline containing 0.3% Tween 20, and incubated 1 h at room temperature with primary antibody specific for rOAT1, rOAT3, P-gp (C219 monoclonal antibody, Signet Laboratories, Dedham, MA, USA), Na^+/K^+-ATPase α-1 subunit (Upstate Biotechnology Inc., Lake Placid, NY, USA) or β-actin (Sigma Chemical Co.). The blots were washed with Tris-buffered saline containing 0.3% Tween 20 and incubated with the secondary antibody (horseradish peroxidase-linked antirabbit immunoglobulin F $(ab)_2$ or horseradish peroxidase-linked anti-mouse immunoglobulin F$(ab)_2$, GE Healthcare Bio-sciences Corp.) for 1 h at room temperature. Immunoblots were visualized with an ECL system (ECL Advance Western Blotting Detection Kit, GE Healthcare Bio-sciences Corp.). The relative amount of each band was determined densitometrically using Densitograph Imaging Software (ATTO Corporation, Tokyo, Japan). Densitometric ratios to control rats were used as the reference and accorded an arbitrary value of 100.

Uptake by rat renal slices

Uptake studies by using isolated rat renal slices were carried out as described in a previous report.[37] Briefly, slices of whole kidney from control and ischemic rats with or without the cobalt intake were stored in ice-cold oxygenated incubation buffer composed of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4, pH7.5. Renal slices were randomly selected and placed for incubation in flasks containing 6 ml of the incubation buffer with [^{14}C]PAH (5 μM, 0.37 kBq/ml) or [^{3}H]ES (5 μM, 1.85 kBq/ml). The uptake of these compounds was carried out at 37°C under an atmosphere of 100% oxygen. [^{3}H]Mannitol (5 μM, 1.85 kBq/ml) was used to calculate the extracellular trapping and nonspecific uptake of [^{14}C]PAH as well as to evaluate the viability of slices. [^{14}C]Mannitol (5 μM, 0.37 kBq/ml) was used for [^{3}H]ES. After incubation for a specified period, the incubation buffer containing radiolabeled compounds was rapidly removed from the flask, washed twice with 5 ml of ice-cold phosphate buffered saline, blotted on filter paper, weighed, and solubilized in 0.5 ml of NCSII (GE Healthcare Bio-sciences Corp.). The amount of radioactivity was then determined in a liquid scintillation counter after adding 5 ml of OCS (GE Healthcare Bio-sciences Corp.).

Statistical analysis

Data were analyzed statistically by analysis of variance and Scheffe's multiple comparison test, unpaired t-test or Mann–Whitney's test. A P-value of less than 0.05 was considered statistically significant.

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Characterization of the Basal Promoter Element of Human Organic Cation Transporter 2 Gene

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ABSTRACT

Human organic cation transporter 2 (hOCT2; SLC22A2) is abundantly expressed in the kidney, and it plays important roles in the renal tubular secretion of cationic drugs. Although the transport characteristics of hOCT2 have been studied extensively, there is no information available for the transcriptional regulation of hOCT2. The present study was undertaken to identify the *cis*-element and *trans*-factor for basal expression of hOCT2. The transcription start site was located 385 nucleotides above the translation start site by using 5′-rapid amplification of cDNA ends. An approximately 4-kilobase fragment of the hOCT2 promoter region was isolated and the promoter activities were measured in the renal epithelial cell line LLC-PK_1. A deletion analysis suggested that the region spanning −91 to −58 base pairs was essential for basal transcriptional activity. This region lacked a TATA-box but contained a CCAAT box and an E-box. Electrophoretic mobility shift assays showed that specific DNA/protein complexes were present in the E-box but not in the CCAAT box, and supershift assays revealed that upstream stimulatory factor 1 (USF-1), which belongs to the basic helix-loop-helix-leucine zipper family of transcription factors, bound to the E-box. Mutation of the E-box resulted in a decrease in hOCT2 promoter activity, and overexpression of USF-1 enhanced the hOCT2 promoter activity in a dose-dependent manner. This article reports the first characterization of the hOCT2 promoter and shows that USF-1 functions as a basal transcriptional regulator of the hOCT2 gene via the E-box.

Numerous organic cations, including endogenous substances, xenobiotics, and metabolites, are excreted from the body. The kidney is critical for the elimination of organic cations, as is the liver, through active secretion via organic cation transport systems. The membrane potential-dependent organic cation transporters (OCTs) are located at the basolateral membrane of renal tubular cells, and they mediate the cellular uptake of cationic compounds from the blood (Koepsell, 1998; Inui et al., 2000; Jonker and Schinkel, 2004). In the brush-border membranes, organic cations are excreted via H^+/organic cation antiport systems, which have been recently identified by Otsuka et al. (2005) and ourselves (Masuda et al., 2006; Terada et al., 2006) as the part of multidrug and toxin extrusion family.

Human (h)OCT2 and hOCT3, but not hOCT1, are expressed in the kidney, and hOCT2 was found to be the most abundant organic cation transporter in the kidney (Motohashi et al., 2002). In addition, recent functional studies revealed that hOCT2 can transport several clinically important compounds such as creatinine (Urakami et al., 2002), the biguanide agent metformin (Kimura et al., 2005), and the anticancer agents cisplatin (Ciarimboli et al., 2005; Yonezawa et al., 2006) and oxaliplatin (Yonezawa et al., 2006). Drug-drug interaction between cetirizine, a new histamine H_1 blocker, and pilsicainide, a new type of antiarrhythmic drug, was also demonstrated to be mediated by OCT2 in patients with renal insufficiency (Tsuruoka et al., 2006).

So far, the pharmacokinetic significance of hOCT2 has been mainly demonstrated by expression and functional transport analyses. Regarding the regulatory aspects, transport activity of hOCT2 is controlled by protein phosphorylation, which is caused by protein kinase C, protein kinase A, phosphatidylinositol 3-kinase, and calcium/calmodulin complex, and the substrate affinity, plasma membrane expression of hOCT2, or both were altered (Çetinkaya et al., 2003; Biermann et al., 2006). In contrast, transcriptional mechanisms of the hOCT2 gene have not been elucidated. Among the human organic ion transporter family, hepatic expression of human organic anion transporter 2 (Popowski et al., 2005)

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ABBREVIATIONS: OCT, organic cation transporter; h, human; USF, upstream stimulatory factor; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; HO, heme oxygenase; SNP, single-nucleotide polymorphism; rSNP, regulatory single-nucleotide polymorphism; WT, wild-type/wild type.

and hOCT1 (Saborowski et al., 2006) was shown to be *trans*-activated by the hepatocyte nuclear factor 4α and suppressed by bile acids via small heterodimer partner. In the kidney, it has been recently demonstrated that the promoter activity of human organic anion transporter 3 is regulated by hepatocyte nuclear factor 1α (Kikuchi et al., 2006), cAMP response element-binding protein-1, and activating transcription factor-1 (Ogasawara et al., 2006).

In the present study, to fully understand the basic transcriptional regulation of hOCT2, we cloned the 5′-flanking region of the hOCT2 gene and identified the minimal region necessary for basal promoter activity. The present results provide direct evidence for the involvement of upstream stimulatory factor (USF)-1, which belongs to the basic helix-loop-helix-leucine zipper family of transcription factors, bound to an E-box, in the regulation of basal promoter activity of hOCT2.

Materials and Methods

Materials. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). T4 kinase and T4 DNA ligase were purchased from Takara Bio (Otsu, Japan). [γ-^{32}P]ATP was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Antibodies used for supershift assays were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Determination of the Putative Transcription Start Site. The putative transcription start sites for hOCT2 were determined by 5′-rapid amplification of cDNA ends (5′-RACE) using Human Kidney Marathon-Ready cDNA (Clontech, Mountain View, CA) according to the manufacturer's instructions. The hOCT2 gene-specific primers for the RACE were designed and synthesized based on the genomic sequence. The 5′-RACE was performed with an adaptor primer 1, which came with the kit and a gene-specific primer of hOCT2. Nested PCR was performed with an adaptor primer 2 and a nested gene-specific primer of hOCT2 (primer sequences are shown in Table 1). 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).

Genomic Cloning of the hOCT2 Promoter. The hOCT2 promoter was isolated from the human genomic DNA (Promega) by a PCR-based method using primers designed based on the human genomic DNA (Table 1). The PCR product was isolated by electrophoresis and subcloned into the firefly luciferase reporter vector, pGL3-Basic (Promega), at KpnI and MluI sites. This full-length reporter plasmid is hereafter referred to as −4261/+23. The transcription factor-binding sites were predicted with TRANSFAC 6.0 software (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi?), with a core similarity of 0.95 and a matrix similarity of 0.90.

Preparation of Deletion Reporter Constructs. The 5′-deleted constructs (−3654/+23, −3140/+23, −2479/+23, −1291/+23, −468/+23, and −411/+23) were generated by digestion of the −4261/+23 construct with KpnI and each of the following enzymes: EcoRI, BstXI, AatII, PstI, StuI, and NsiI, respectively. The ends were blunted with T4 DNA polymerase (Takara Bio) and then self-ligated. The other constructs were generated by PCR with primers containing a KpnI site and a MluI site (Table 1). The site-directed mutations in the putative E-box were introduced into the −91/+23 construct with a QuikChange II site-directed mutagenesis kit (Stratagene, La

TABLE 1
Oligonucleotide sequences of primers

Name	Sequence	Position
5′-RACE		NM_003058
Gene-specific primer	5′-CGGCCAAACCTGTCTGCTATGTAGCCG-3′	697/671
Nested gene-specific primer	5′-TCCATGCTCCAGGACATCGTCCACGGT-3′	207/180
hOCT2 cloning		NT_007422.12
hOCT2 −4261	5′-GGGGTACCCAGGAGCCTTCATTCAGAATG-3′	−4261/−4239
hOCT2 −294	5′-GGGGTACCGACGGCTCTTGTTGTTGGTTG-3′	−294/−273
hOCT2 −150	5′-GGGGTACCATCCTAAGGCTCACGGCCAAC-3′	−150/−121
hOCT2 −91	5′-GGGGTACCGCCTTGTGGCCAAACACGTGT-3′	−91/−70
hOCT2 −58	5′-GGGGTACCGCCTTGAAGAAAAGCTGGCG-3′	−58/−39
hOCT2 −40	5′-GGGCTAGTTCTCCATAGGGCCTTGAAG-3′	−40/−19
hOCT2 −18	5′-GGGGTACCATTAAGTTCCTGGCTGCTCG-3′	−18/−3
hOCT2 +23	5′-CGACGCGTTCACAGCCCAGTAATCTTCCC-3′	1/23
USF-1 cloning		NM_007122
Forward	5′-TCGGGAATTCCCCCTCACAGAGAGATGAAGGGG-3′	97/129
Reverse	5′-GATCCCTCGAGTTAGTTGCTGTCATTCTTGATGACG-3′	1029/1053
EMSA probe		
Collagen gene		Nagato et al. (2004)
Forward	5′-AAGAGATTAACCAATCACGTACGGTCT-3′	
Reverse	5′-AGACCGTACGTGATTGGTTAATCTCTT-3′	
Collagen mutCCAAT		Nagato et al. (2004)
Forward	5′-AAGAGATTAATCTAGCACGTACGGTCT-3′	
Reverse	5′-AGACCGTACGTGCTAGATTAATCTCTT-3′	
HO-1		Hock et al. (2004)
Forward	5′-GCCTTGTGGCCAAACACGTGTGTTTTCT-3′	
Reverse	5′-AGAAAACACAGCTGTTTGGCCACAAGGC-3′	
HO-1 mutE-box		
Forward	5′-GCCTTGTGGCCAAACATTTGTGTTTTCT-3′	
Reverse	5′-AGAAAACACAAATGTTTGGCCACAAGGC-3′	
hOCT2		NT_007422.12
Forward	5′-GCCTTGTGGCCAAACACGTGTGTTTTCT-3′	−101/−74
Reverse	5′-AGAAAACACACGTGTTTGGCCACAAGGC-3′	−101/−74
hOCT2 mutCCAAT		NT_007422.12
Forward	5′-GCCTTGTGGTCTAGCACGTGTGTTTTCT-3′	−101/−74
Reverse	5′-AGAAAACACACGTGCTAGACCACAAGGC-3′	−101/−74
hOCT2 mutE-box		NT_007422.12
Forward	5′-GCCTTGTGGCCAAACATTTGTGTTTTCT-3′	−101/−74
Reverse	5′-AGAAAACACAAATGTTTGGCCACAAGGC-3′	−101/−74

Jolla, CA) with the primers listed in Table 1. The nucleotide sequences of these deleted or mutated constructs were verified.

Cloning of hUSF-1 cDNA. cDNA for hUSF-1 (accession no. NM_007122) was isolated from Human Kidney Marathon-Ready cDNA (Clontech). Primers are listed in Table 1. The PCR product was subcloned into the expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA), and its sequence was verified.

Cell Culture and Luciferase Assay. The porcine kidney epithelial cell line LLC-PK_1 was obtained from American Type Culture Collection (ATCC CRL-1392; Manassas, VA). Cell culture, transfection, and the luciferase assay were performed as described previously (Asaka et al., 2006).

Electrophoretic Mobility Shift Assay. Nuclear extract was prepared from LLC-PK_1 cells according to the method of Shimakura et al. (2005). The probes listed in Table 1 were prepared by annealing complementary sense and antisense oligonucleotides, followed by end-labeling with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara Bio) and purification through a Sephadex G-25 column (GE Healthcare). The binding mixture consisted of 10 μg of LLC-PK_1 nuclear extract and unlabeled competitor probes in a buffer solution containing 120 mM KCl, 20 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol, 10 mM NaF, 0.1 mM Na_3VO_4, 5% mM glycerol, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, and 2% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After preincubation for 30 min, labeled probes (~0.4 ng) were added, and the binding mixture was incubated for a further 30 min. For supershift assays, 1 μg of USF-1 antibody (sc-8983X) or USF-2 antibody (sc-862X) was added 30 min before the addition of the labeled probe. The volume of the binding mixture was 20 μl throughout the experiment. The DNA-protein complex was then separated on a 4% polyacrylamide gel at room temperature in 0.5× Tris borate-EDTA buffer. The gels were dried and exposed to X-ray film for autoradiography.

Data Analysis. The results were expressed relative to the pGL3-Basic vector set, and they represent the means ± S.E. of three replicates. Two or three experiments were conducted, and representative results are shown. Data were analyzed statically by the Student's *t* test or by the one-way analysis of variance followed by Dunnett's test.

Results

Determination of the Transcription Start Site for hOCT2 in Human Kidney Using 5′-RACE. The transcription start site for hOCT2 in the human kidney was identified by 5′-RACE. The putative start site was determined using the longest RACE product. Sequencing of the amplified bands revealed that the terminal position of hOCT2 cDNA with the longest 5′-untranslated region was located 385 nucleotides above the translation start site, which is 243 base pairs upstream of the 5′-end of hOCT2 cDNA reported previously (Gorboulev et al., 1997). So, the terminal position of hOCT2 cDNA with the longest 5′-untranslated region was numbered +1 as the transcription start site in this study.

Isolation and Analysis of the 5′-Flanking Region of the hOCT2 Gene. Based on the result of 5′-RACE, approximately 4 kilobases of the 5′-flanking region of the hOCT2 gene were isolated and subcloned into pGL3-Basic. Promoter activity was assessed in LLC-PK_1 cells, because this cell line has active organic cation transport systems (Saito et al., 1992), and it shows promoter activity of rat OCT2 (Asaka et al., 2006). To determine the minimal region required for basal activity of the promoter, a series of deletion constructs were transfected into LLC-PK_1 cells, and luciferase activity was measured (Fig. 1). The longest reporter construct

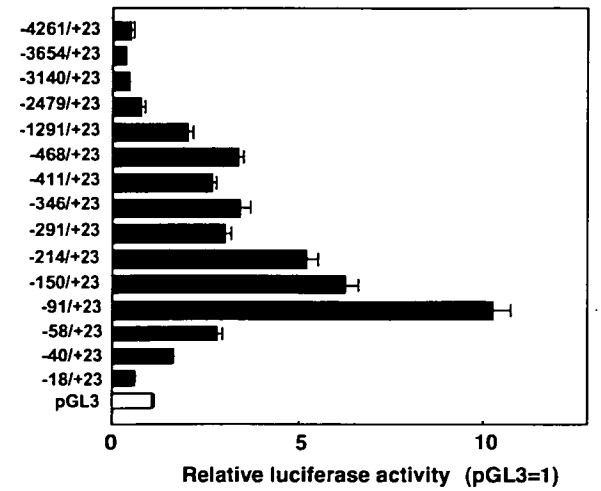

Fig. 1. Identification of transcriptional activity and deletion analysis of the hOCT2 promoter in LLC-PK_1 cells. A series of deleted promoter constructs [equimolar amounts of the −4261/+23 construct (600 ng)] were transfected into LLC-PK_1 cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative -fold increase compared with pGL3-Basic vector and represent the means ± S.E. of three replicates. This figure is a representative one from three separate experiments.

(−4261/+23) had the same level of luciferase activity as the control vector pGL3-Basic. The level of luciferase activity gradually increased as the region spanning from −4261 to −91 was deleted, and the reporter construct (−91/+23) had approximately 10-fold more luciferase activity than pGL3-Basic. In contrast, the constructs (−58/+23, −40/+23, and −18/+23) exhibited little luciferase activity. These results suggested that the region between −91 and −58 contained positive *cis*-acting elements for efficient basal expression of the hOCT2 gene. Figure 2 shows a computational analysis of the −106/−58 region of the hOCT2 promoter. Using TRANSFAC 6.0 (www.gene-regulation.com/), we found a putative CCAAT box (5′-CCAAA-3′) and E-box (5′-CACGTG-3′) in this region. The CCAAT box can be recognized by several transcription factors such as CCAAT/enhancer-binding proteins (Ramji and Foka, 2002) and nuclear factor-Y (Mantovani, 1999), and the E-box is stimulated by USFs (Corre and Galibert, 2005). These transcription factors were reported to help maintain the basal promoter activities of various genes.

Electrophoretic Mobility Shift Assay. To confirm which *cis*-element was involved in the basal promoter activity, EMSA was performed using nuclear extract from LLC-PK_1 cells and the hOCT2 probe (−101/−74), which contains both a CCAAT box and an E-box. At first, we tested the involvement of the CCAAT box. For a positive control of the

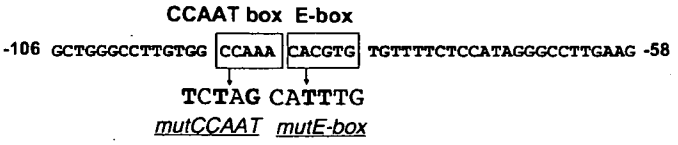

Fig. 2. Nucleotide sequence of the promoter region (−106 to −58) that had the highest level of basal activity. Numbering is relative to the transcription start site. Putative binding sites for the transcription factors are indicated on the sequence. Site-directed mutations that destroy putative transcription factor-binding elements were introduced individually and designated *mutCCAAT* and *mut E-box*. The nucleotides altered for the mutational analysis are shown in bold under the wild-type (WT) sequence.

EMSA for the CCAAT box, an oligonucleotide of the CCAAT box derived from the human pro-α3(V) collagen gene (Nagato et al., 2004) was used. As shown in Fig. 3, a specific DNA-protein complex was observed using the pro-α3(V) collagen gene CCAAT box probe (lane 2). In the same buffer, the hOCT2 (−101/−74) probe formed a DNA-protein complex (lane 6), but this was prevented by the hOCT2 probe with mutCCAAT, which lacks a CCAAT box (lane 8). Furthermore, the consensus sequence for the CCAAT box did not prevent the formation of the complex (lane 9). These results suggested that nuclear extracts did not bind to the CCAAT box.

We then examined the contribution of the E-box. For a positive control, a oligonucleotide for the E-box derived from the heme oxygenase (HO)-1 promoter (Hock et al., 2004) was used. As shown in Fig. 4, a specific DNA-protein complex was observed using the HO-1 gene E-box probe (lane 2), and this complex was supershifted using anti USF-1 antibody (lane 3). In these conditions, the hOCT2 probe also formed a DNA-protein complex (lane 6). The formation of this complex was completely prevented by the addition of an excess amount of unlabeled oligonucleotide for the hOCT2 probe (lane 7), hOCT2 mutCCAAT (lane 9), and the consensus sequence for the E-box (lane 10), but not by mutE-box lacking an E-box (lane 8). Furthermore, anti-USF-1 antibody, but not anti USF-2 antibody, was able to supershift the DNA-protein complex (lanes 11 and 12). These results indicated that USF-1 bound to the E-box of the hOCT2 promoter.

Mutagenesis of the E-box. To confirm the functional importance of the E-box, mutations were introduced into the −150/+23 and −91/+23 constructs and their promoter activities were examined. As shown in Fig. 5, both constructs lost all luciferase activity compared with the wild type. These results suggest that the E-box is responsible for the basal promoter activity of hOCT2.

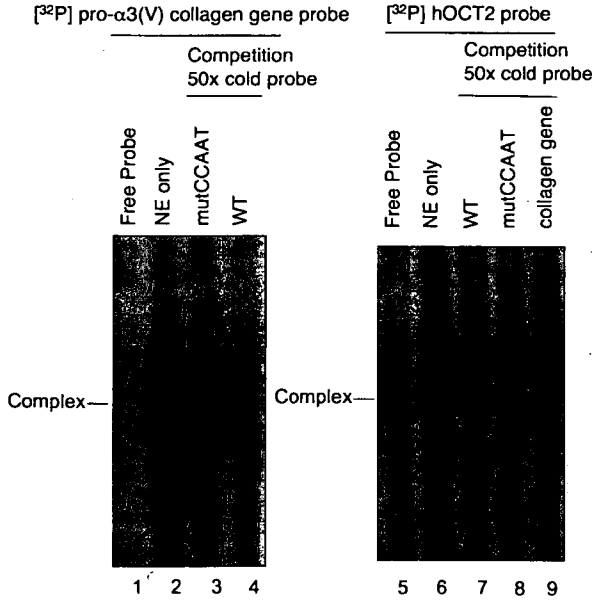

Fig. 3. EMSA of LLC-PK$_1$ nuclear proteins binding to the probes containing putative CCAAT box-binding elements. Nuclear extract from LLC-PK$_1$ cells was incubated with the ^{32}P-labeled oligonucleotide probes [pro 3α(V) collagen gene probe and hOCT2 probe] alone (lanes 2 and 6) or in the presence of excess unlabeled WT oligonucleotide (lanes 4 and 7), mutated oligonucleotide (lanes 3 and 8), or pro 3α(V) collagen gene oligonucleotide (lane 9). In lanes 1 and 5, nuclear extract was not added.

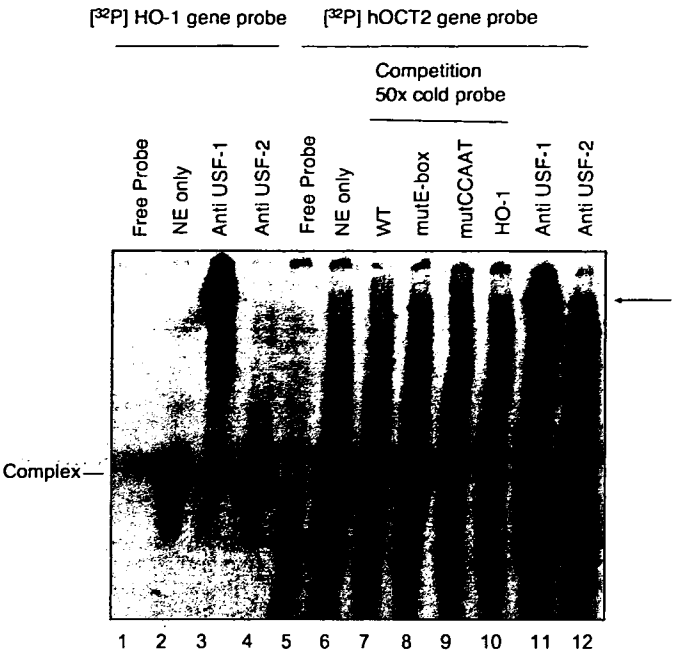

Fig. 4. EMSA of LLC-PK$_1$ nuclear proteins binding to the probes containing putative E-box binding elements. Nuclear extract from LLC-PK$_1$ cells was incubated with the ^{32}P-labeled oligonucleotide probes (heme oxygenase-1 gene probe and hOCT2 probe) alone (lanes 2 and 6) or in the presence of excess unlabeled WT oligonucleotide (lane 7), E-box mutated oligonucleotide (lane 8), CCAAT box mutated oligonucleotide (lane 9), HO-1 gene oligonucleotide (lane 10), anti USF-1 antibody (lanes 3 and 11), and anti USF-2 antibody (lanes 4 and 12). In lanes 1 and 5, nuclear extract was not added. An arrow indicates the supershifted complexes.

Transactivation of the Promoter Activity by Overexpression of USF-1. Finally, we investigated the effect of the overexpression of USF-1 on the promoter activity of hOCT2 (Fig. 6). The −91/+23 construct was cotransfected into LLC-PK$_1$ cells with the USF-1 expression vector. The promoter activity of hOCT2 showed a dose-dependent increase on the coexpression of USF-1, providing direct evidence that USF-1 enhanced the promoter activity.

Discussion

In the present study, we performed a functional promoter assay of hOCT2, and we obtained convincing evidence of the involvement of USF-1 bound to an E-box in the regulation of hOCT2 basal expression. This conclusion is supported by results of experiments involving EMSAs, mutagenesis of the E-box, and overexpression of USF-1. USF-1 was originally described as a transcription factor derived from HeLa nuclear extract that binds to an E-box of the adenovirus major late promoter (Sawadogo and Roeder, 1985). Subsequent analysis revealed that this factor is involved in the basal transcriptional regulation of various genes, including the human angiotensinogen (Yanai et al., 1997), HO-1 (Hock et al., 2004), and prolyl-4-hydroxylase (I) (Chen et al., 2006) genes. Furthermore, USF acts not only as a classical upstream activator but also as a factor that interacts with initiator elements of a variety of core promoters, which can lead to markedly enhanced levels of basal transcription (Corre and Galibert, 2005). The core E-box sequence CANNTG is usually conserved, with the two central sequence nucleotides (NN), in most cases, either GC or CG (Corre and Galibert, 2005). Because the E-box of hOCT2 is

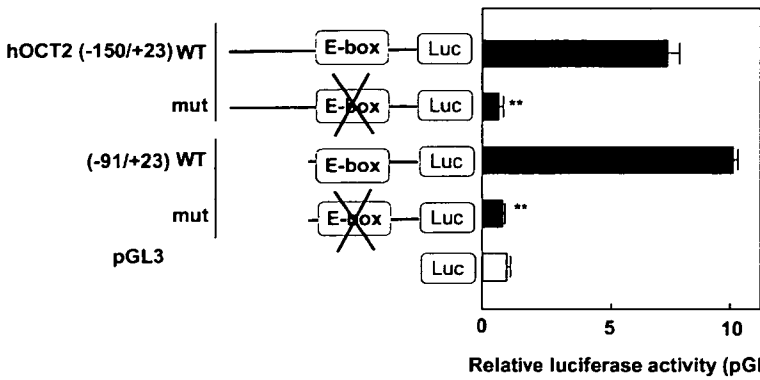

Fig. 5. Mutational analysis of the putative E-box binding elements of the hOCT2 promoter. The mutated −150/+23 and −91/+23 constructs (600 ng) were transiently expressed in LLC-PK$_1$ cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative -fold increase compared with pGL3-Basic vector and represent the means ± S.E. of three replicates. **, $P < 0.01$, significantly different from WT. This figure is a representative one from three separate experiments.

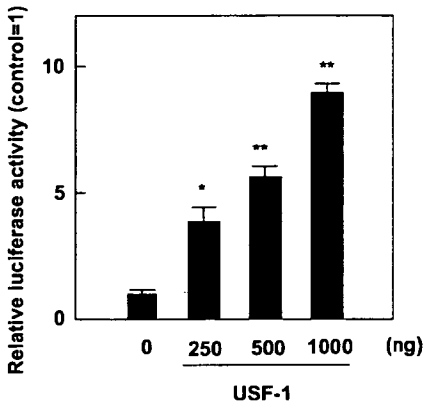

Fig. 6. Effect of hUSF-1 overexpression on hOCT2 transcriptional activity. LLC-PK$_1$ cells were transiently transfected with 250 ng of the −91/+23 construct and 250, 500, and 1000 ng of the hUSF-1 expression vector. The total amount of transfected DNA was kept constant by adding empty vector. Data are reported as the relative -fold increase compared with no hUSF-1 and represent the means ± S.E. of three replicates. *, $P < 0.05$, significantly different from control (no USF-1). **, $P < 0.01$, significantly different from control. This figure is a representative one from three separate experiments.

CACGTG, a perfectly conserved consensus sequence, it is also reasonable that USF-1 binds to the E-box of the hOCT2 promoter region.

USF-1 is a key regulator of a number of gene regulatory networks, including the stress and immune responses, cell cycle and proliferation, and lipid and glucid metabolism (Corre and Galibert, 2005). Familial combined hyperlipidemia, characterized by elevated levels of serum total cholesterol, triglycerides, or both, is associated with the USF-1 haplotype (Pajukanta et al., 2004). Recently, it was reported that USF-1 gene polymorphisms are associated with increased sensitivity to antilipolytic insulin (Kantartzis et al., 2007). In human kidney, the mRNA level of hOCT2 was highest of any of the OCT family (Motohashi et al., 2002), suggesting that hOCT2 has a more important role in the transport of organic cations across the basolateral membrane than other members of the hOCT family in the proximal tubules. However, the expression pattern of renal drug transporters was reported to vary among patients (Terada and Inui, 2007). This variation may be caused by single-nucleotide polymorphisms (SNPs) in the hOCT2 promoter region or USF-1 coding region.

Regulatory SNPs (rSNPs), which lie outside of the amino acid coding regions of genes, affect the regulation of gene expression. Recent research suggests that approximately 50% of genes have one or more common rSNPs associated with gene expression (Buckland, 2006). For example, the uridine diphosphate glucuronosyltransferase UGT1A1*28 polymorphism, characterized by an extra TA repeat in the promoter region of the gene, is involved in the toxicity of irinotecan (Iyer et al., 2002). Thiopurine *S*-methyltransferase, which is required to metabolize thiopurine, has a variable number tandem repeats in the 5′-flanking region involved in the gene expression (Alves et al., 2001). In addition, it has been reported that a rSNP of *SLC22A5* (*OCTN2*) affects the transcription of OCTN2, contributing to the pathogenesis of Crohn's disease (Peltekova et al., 2004). A rSNP within the E-box core motif also modulates gene regulation. For example, a single base transition within the USF E-box consensus sequence of the thymidylate synthase gene, implicated in the metabolism of folate, prevents the USF complex from binding to its cognate sequence (Mandola et al., 2003). It is unknown whether SNPs exist in the hOCT2 gene E-box, but it is possible that these SNPs affect the pharmacokinetics of cationic drugs.

USF-1 is a ubiquitously expressed transcription factor (Sirito et al., 1994), but hOCT2 is mainly expressed in the kidney (Urakami et al., 2002). The mechanism of this tissue-specific expression has not been clarified yet. The E-box of the hibernation-specific protein HP-27 is hypomethylated in the liver, but it is highly methylated in the kidney and heart, being involved in liver-specific expression (Fujii et al., 2006). Kidney-specific transcription may be controlled by methylation of the E-box in the kidney or an unidentified kidney-specific transcription factor.

In conclusion, the present results indicate that USF-1 functions as a basal transcriptional regulator of the hOCT2 gene, making this study the first to identify the *cis*-elements and *trans*-factors necessary for the regulation of hOCT2. These findings should serve as a basis for future investigations into the molecular regulation of the transport of organic cations and some pharmaceuticals in the human kidney.

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Identification of Essential Histidine and Cysteine Residues of the H^+/Organic Cation Antiporter Multidrug and Toxin Extrusion (MATE)[S]

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ABSTRACT

Multidrug and toxin extrusion 1 (MATE1) has been isolated as an H^+/organic cation antiporter located at the renal brush-border membranes. Previous studies using rat renal brush-border membrane vesicles indicated that cysteine and histidine residues played critical roles in H^+/organic cation antiport activity. In the present study, essential histidine and cysteine residues of MATE1 family were elucidated. When 7 histidine and 12 cysteine residues of rat (r)MATE1 conserved among species were mutated, substitution of His-385, Cys-62, and Cys-126 led to a significant loss of tetraethylammonium (TEA) transport activity. Cell surface biotinylation and immunofluorescence analyses with confocal microscopy indicated that rMATE1 mutant proteins were localized at plasma membranes. Mutation of the corresponding residues in human (h)MATE1 and hMATE2-K also diminished the transport activity. The transport of TEA via rMATE1 was inhibited by the sulfhydryl reagent *p*-chloromercuribenzenesulfonate (PCMBS) and the histidine residue modifier diethyl pyrocarbonate (DEPC) in a concentration-dependent manner. The PCMBS-caused inhibition of the transport via rMATE1 was protected by an excess of various organic cations such as TEA, suggesting that cysteine residues act as substrate-binding sites. In the case of DEPC, no such protective effects were observed. These results suggest that histidine and cysteine residues are required for MATE1 to function and that cysteine residues may serve as substrate-recognition sites.

Proximal tubules play important roles in the renal elimination of drugs. Cationic drugs are secreted from blood to urine by the combined efforts of two distinct classes of organic cation transporters: one driven by the transmembrane electrical potential difference in the basolateral membranes, and the other driven by the transmembrane H^+ gradient in the brush-border membranes (Inui and Okuda, 1998). To date, three kinds of membrane potential-dependent organic cation transporters (OCT1~3) have been identified and characterized (Burckhardt and Wolff, 2000; Inui et al., 2000; Wright, 2005).

In contrast to OCTs, the molecular nature of H^+/organic cation antiport system has not been characterized, but recently, orthologs of the multidrug and toxin extrusion (MATE) family have been identified in various species (Otsuka et al., 2005a; Hiasa et al., 2006; Masuda et al., 2006; Terada et al., 2006). We have cloned rat (r)MATE1 cDNA and demonstrated that rMATE1 mRNA is mainly expressed in the kidney (proximal convoluted and straight tubules), and rMATE1 can transport not only organic cations such as tetraethylammonium (TEA), cimetidine, and metformin but also the zwitterionic compound cephalexin (Terada et al., 2006). Furthermore, we revealed recently that an oppositely directed H^+ gradient serves as a driving force for the transport of TEA via rMATE1 (Tsuda et al., 2007). We also cloned human (h)MATE2-K cDNA and revealed that hMATE2-K and the hMATE1 was localized at the brush-border membranes of renal proximal tubules (Masuda et al., 2006). hMATE2-K can also transport organic cations such as TEA, procainamide, metformin, and creatinine and works as an H^+/organic cation antiporter (Masuda et al., 2006). These studies suggested that the mammalian MATE family showed similar characteristics to the renal H^+/organic cation antiport system (Inui and Okuda, 1998; Wright, 2005). During

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ABBREVIATIONS: MATE, multidrug and toxin extrusion; TEA, tetraethylammonium; DEPC, diethyl pyrocarbonate; PCMBS, *p*-chloromercuribenzenesulfonate; OCT, organic cation transporter; PAH, *p*-aminohippurate; TBS, Tris-buffered saline; HEK, human embryonic kidney.

the course of studying the transport of TEA using rat renal brush-border membrane vesicles, we found that treatment of membrane vesicles with sulfhydryl reagents such as *p*-chloromercuribenzenesulfonate (PCMBS) (Hori et al., 1987) and the histidine residue modifier diethyl pyrocarbonate (DEPC) (Hori et al., 1989) significantly inhibited [^{14}C]TEA transport by H^+/organic cation antiport system. Furthermore, very recently, Ohta et al. (2006) demonstrated that PCMBS inhibited the uptake of TEA by rMATE1. Based on these findings, it was speculated that histidine and cysteine residues play important roles in the transport activity of MATE family. The present study was undertaken to identify the essential histidine and cysteine residues of the MATE family (especially rMATE1) using site-directed mutagenesis and to examine their functional roles using chemical modifiers.

Materials and Methods

Materials. Cephalexin was donated by Shionogi Co. (Osaka, Japan). [^{14}C]TEA (2.035 GBq/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). DEPC, cimetidine, and TEA were obtained from Nacalai Tesque (Kyoto, Japan). PCMBS, metformin, and *p*-aminohippurate (PAH) were purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available.

Plasmids and Site-Directed Mutagenesis. The rMATE1 cDNA was excised from rMATE1/pcDNA3.1 (Terada et al., 2006), and was subcloned into pFLAG-CMV-6 (Sigma) to yield FLAG-rMATE1. The site-directed mutations of histidine or cysteine residues were introduced into FLAG-rMATE1, hMATE1/pcDNA3.1 (Yonezawa et al., 2006), or hMATE2-K/pcDNA3.1 (Masuda et al., 2006) with a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers listed in Supplemental Table S1. The nucleotide sequences of these constructs were confirmed using multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

Cell Culture, Transfection, and Transport Measurements. HEK293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) were cultured as described previously (Urakami et al., 2002; Terada et al., 2006). Various constructs were transfected into HEK293 cells using LipofectAMINE 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At 48 h after the transfection, the cells were used for uptake experiments. Cellular uptake of [^{14}C]TEA was measured with monolayers grown on poly(D-lysine)-coated 24-well plates. To manipulate the intracellular pH, intracellular acidification was performed by pretreatment with ammonium chloride (30 mM, 20 min at 37°C, pH 7.4) (Masuda et al., 2006; Terada et al., 2006). The medium was then removed, and 0.2 ml of incubation medium, pH 7.4, containing [^{14}C]TEA was added. The medium was aspirated off at the end of the incubation, and monolayers were rapidly rinsed 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. 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.

Pretreatment with DEPC and PCMBS. HEK293 cells were washed twice with incubation medium, pH 7.4. Monolayers were then incubated with DEPC, pH 6.0, in phosphate-buffered saline for 10 min at 25°C or PCMBS, pH 7.4, in incubation medium for 10 min at 25°C. Then cells were washed twice before intracellular acidification.

Cell Surface Biotinylation. Cell surface biotinylation was performed according to the methods of Hong et al. (2004) with some modification. HEK293 cells were grown on poly(D-lysine)-coated six-well plates and transfected with the rMATE1cDNAs. At 48 h after the transfection, cells were washed with ice-cold phosphate-buffered saline calcium/magnesium (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.3) and then treated with 1 ml of membrane-impermeable biotinylating agent, sulfo-NHS-SS-biotin (Pierce, Rockford, IL) (1.5 mg/ml) at 4°C for 1 h. Subsequently, the cells were washed three times with ice-cold phosphate-buffered saline calcium/magnesium containing 100 mM glycine and then incubated for 20 min at 4°C with the same buffer to remove the remaining labeling agent. After washing with phosphate-buffered saline calcium/magnesium, cells were disrupted with 700 μl of lysis buffer (10 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 7.4) containing protease inhibitors at 4°C for 1 h with constant agitation. After centrifugation, 140 μl of streptavidin agarose beads (Pierce) were added to 600 μl of cell lysate and incubated for 1 h at room temperature to isolate the plasma membrane protein.

Western Blot Analysis. The procedures for Western blot analysis were described previously (Terada et al., 1996). Monoclonal anti-FLAG-M2 antibody (1:4000 dilution; Sigma) or Na^+/K^+-ATPase antibody (1:2000 dilution; Upstate Biotechnology, Lake Placid, NY) was used as the primary antibody. A peroxidase-conjugated anti-mouse IgG antibody was used for detection of bound antibodies, and strips of the blots were visualized by chemiluminescence on X-ray film.

Immunofluorescence of Transfected Cells. HEK293 cells were seeded onto poly(D-lysine)-coated cover glasses (BD Biosciences, San Jose, CA), and then transfection was performed. Cells were washed twice in Tris-buffered saline (TBS), fixed for 1 min at room temperature in a mixture of methanol/acetone (1:1), and rewashed in TBS. The cells were incubated for 1 h at room temperature in TBS containing monoclonal anti-FLAG M2-FITC antibody (Sigma) (1:1000). Cells were thoroughly washed, and coverslips were mounted. These samples were examined with Eclipse E800 fluores-

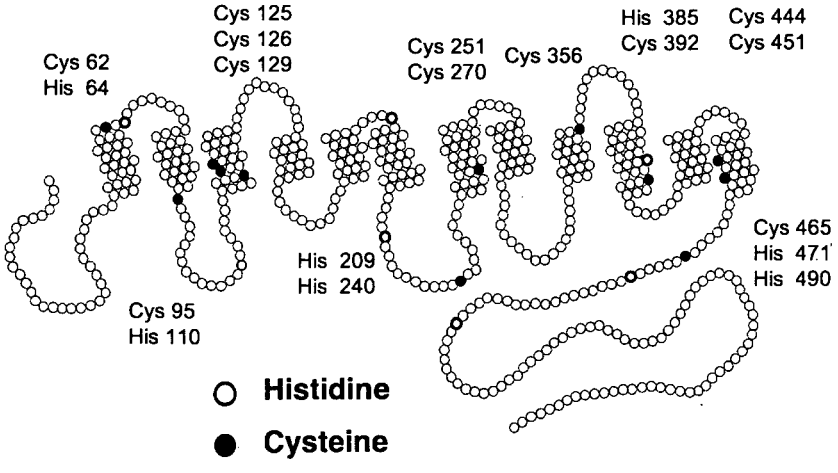

Fig. 1. Putative secondary structure of rMATE1 protein. The histidine (○) and cysteine (●) residues conserved among species are shown.

cence microscope (Nikon, Tokyo, Japan) equipped with MRC-1024 laser confocal system (Bio-Rad Laboratories).

Statistical Analysis. The significance of differences between the wild-type and mutant were analyzed using Dunnett's post hoc analysis. Two or three experiments were conducted, and representative results are shown. Other analyses were conducted with Student's *t* test.

Results

Transport Analysis of rMATE1 Histidine and Cysteine Mutants. When amino acid sequences of rat, human, and mouse MATE1 were compared, 7 histidine and 12 cysteine residues were found to be conserved. Figure 1 shows the location of these amino acids in the predicted secondary structure of rMATE1. To determine which residues are essential for the transport activity of rMATE1, conserved histidine and cysteine residues were changed to glutamine and glycine residues, respectively. As shown in Fig. 2A, [^{14}C]TEA uptake was significantly reduced only in the rMATE1 H385Q among histidine mutants. As for cysteine mutants, the transport activity of C62G and C126G mutants was remarkably reduced, and [^{14}C]TEA uptake by C129G, C356G, C392G, and C451G mutants was significantly but modestly inhibited (Fig. 2B). We then focused on the amino acid residues His-385, Cys-62, and Cys-126 and further evaluated their functional importance. The substitution of these three amino acid residues in rMATE1 for other amino acid residue also abolished [^{14}C]TEA uptake (Fig. 3). These three amino acid residues are also conserved in hMATE1 (His-386, Cys-63, and Cys-127) and hMATE2-K (His-382, Cys-59, and Cys-123), and therefore, histidine and cysteine mutants of hMATE1 and hMATE2-K were prepared. As shown in Fig. 4, the transport of [^{14}C]TEA via the hMATE1 and hMATE2-K mutants was also diminished, suggesting that these histidine and cysteine amino acid residues are essential to the MATE families.

Protein Expression of rMATE1 Mutants. One possible reason for the defective transport activity of these mutants is a decreased level of the mutant protein in the plasma membranes of HEK293 cells, which could be caused by reduced stability and/or impaired insertion into the membranes of the mutants. To examine this possibility, Western blot analyses of plasma membranes prepared from each rMATE1 mutant and the immunolocalization of rMATE1 mutants were performed. Cell surface biotinylation was performed to specifically capture plasma membranes, and biotinylation techniques were confirmed to detect Na^+/K^+-ATPase for all samples (Goel et al., 2005). As shown in Fig. 5, all MATE1

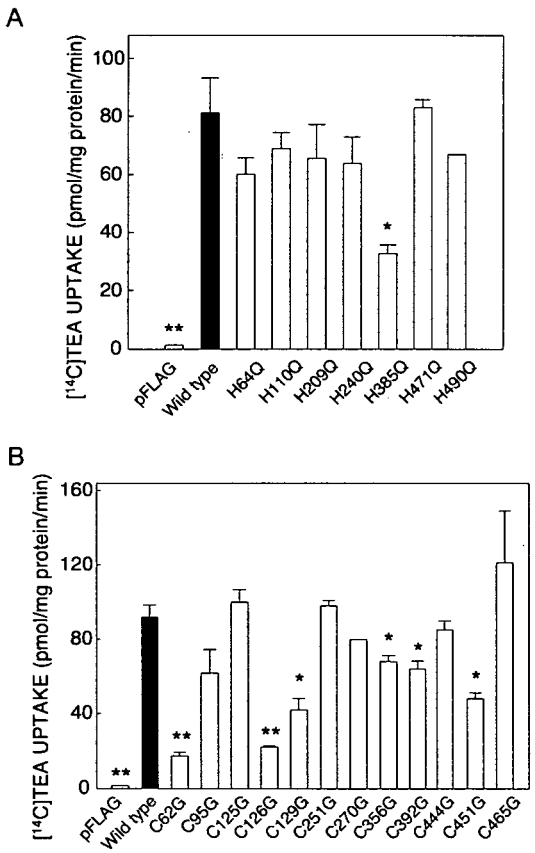

Fig. 2. Uptake of [^{14}C]TEA by HEK293 cells expressing wild-type and histidine (A) or cysteine (B) mutants of rMATE1. The cells were preincubated with incubation medium, pH 7.4, in the presence of 30 mM ammonium chloride for 20 min. Then the preincubation medium was removed, and the cells were incubated with 5 μM [^{14}C]TEA (10.36 kBq/ml, pH 7.4) for 1 min at 37°C. Each column represents the mean ± S.E. of three monolayers. This figure is a representative one from three separate experiments. *, $p < 0.05$, **, $p < 0.01$, significantly different from the wild type.

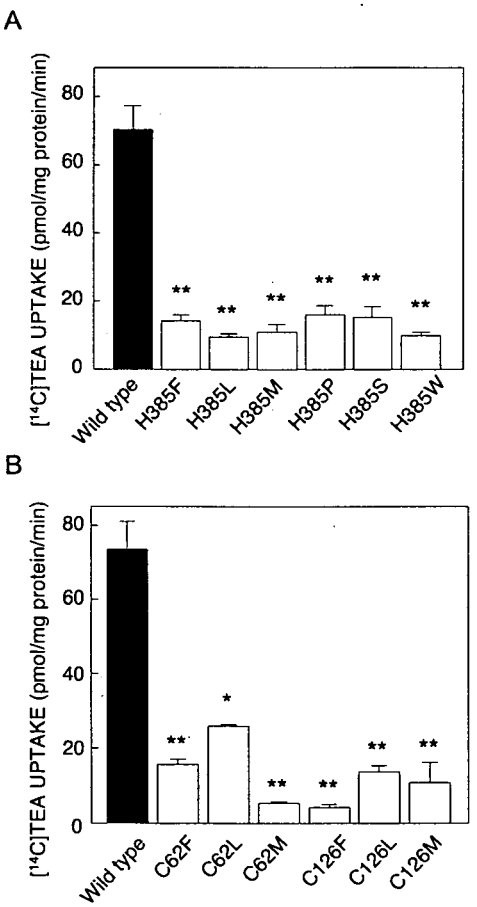

Fig. 3. Effect of various amino acid substitutions of His-385 (A), Cys-62, and Cys-126 (B) of rMATE1 on the uptake of [^{14}C]TEA by HEK293 cells expressing each rMATE1 mutant. The cells were preincubated with incubation medium, pH 7.4, in the presence of 30 mM ammonium chloride for 20 min. Then the preincubation medium was removed, and the cells were incubated with 5 μM [^{14}C]TEA, pH 7.4, for 1 min at 37°C. Each column represents the mean ± S.E. for three monolayers. This figure is a representative one from three separate experiments. *, $p < 0.05$, **, $p < 0.01$, significantly different from the wild type.

mutant proteins were expressed at plasma membranes and wild-type MATE1. Furthermore, immunofluorescence analyses with confocal microscopy revealed that the rMATE1 mutant proteins with H385Q, C62G, and C126G were localized at the plasma membranes (Fig. 6). Although intracellular staining in addition to membrane labeling was observed, this might be caused by the overexpression of rMATE1. This issue of expression pattern could not be ruled out in the transient expression system. These findings suggested that the low levels of transport activity of rMATE1 mutants with H385G, C62G, and C126G were not caused by the alteration of protein expression in plasma membranes.

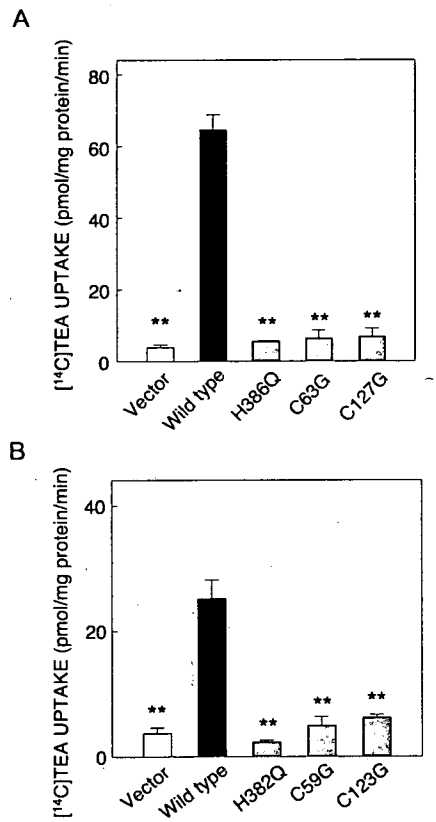

Fig. 4. Uptake of [^{14}C]TEA by HEK293 cells expressing wild-type and histidine and cysteine mutants of hMATE1 (A) or hMATE2-K (B). The cells were preincubated with incubation medium, pH 7.4, in the presence of 30 mM ammonium chloride for 20 min. Then the preincubation medium was removed, and the cells were incubated with 5 μM [^{14}C]TEA, pH 7.4, for 1 min at 37°C. Each column represents the mean ± S.E. of three monolayers. This figure is a representative one from two separate experiments. **, $p < 0.01$, significantly different from wild type.

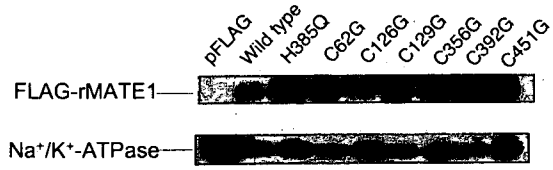

Fig. 5. Western blot analysis of plasma membranes obtained from HEK293 cells transiently expressing wild-type rMATE1 or histidine or cysteine mutants of rMATE1. Plasma membrane fractions prepared by cell surface biotinylation were separated by SDS-polyacrylamide gel electrophoresis (10%) and blotted onto polyvinylidene difluoride membranes. Monoclonal anti-FLAG-M2 antibody or Na^+/K^+-ATPase antibody was used as a primary antibody. A horseradish peroxidase-conjugated anti-mouse IgG antibody was used for detection of the bound antibody, and the strips of blots were visualized by chemiluminescence on X-ray film.

Effects of DEPC or PCMBS Treatment on rMATE1 Function. We next examined the functional roles of the cysteine and histidine residues by using chemical modifiers such as the sulfhydryl reagent PCMBS and the histidine residue modifier DEPC. As shown in Fig. 7, pretreatment of rMATE1-expressing cells with PCMBS or DEPC led to a concentration-dependent decrease in the transport of [^{14}C]TEA. The half-maximal inhibition for [^{14}C]TEA transport via rMATE1 was calculated as 1.13 ± 0.91 mM for DEPC and 37.2 ± 10.2 μM for PCMBS.

Effect of DEPC or PCMBS in the Presence of Unlabeled TEA on rMATE1. We examined the effect of DEPC or PCMBS pretreatment in the presence of unlabeled TEA on rMATE1 function. As shown in Fig. 8A, unlabeled TEA had no effect on the inhibition of [^{14}C]TEA uptake by DEPC. On the other hand, unlabeled TEA protected against the inhibition of [^{14}C]TEA uptake caused by PCMBS pretreatment (Fig. 8B). The PCMBS-caused inhibition of [^{14}C]TEA transport via rMATE1 was also blocked by an excess of other MATE1 substrates such as cephalexin, cimetidine, and metformin but not by a typical organic anion, PAH. The results suggest that cysteine residues in rMATE1 interact with the substrates.

We further examined the protective effect of unlabeled TEA on [^{14}C]TEA uptake by C62G and C126G mutants treated with PCMBS (0.1 mM). [^{14}C]TEA uptake by C62G or C126G mutant was significantly decreased by the pretreatment of PCMBS (C62G: 7.52 ± 1.68 to 1.68 ± 0.13 pmol/mg

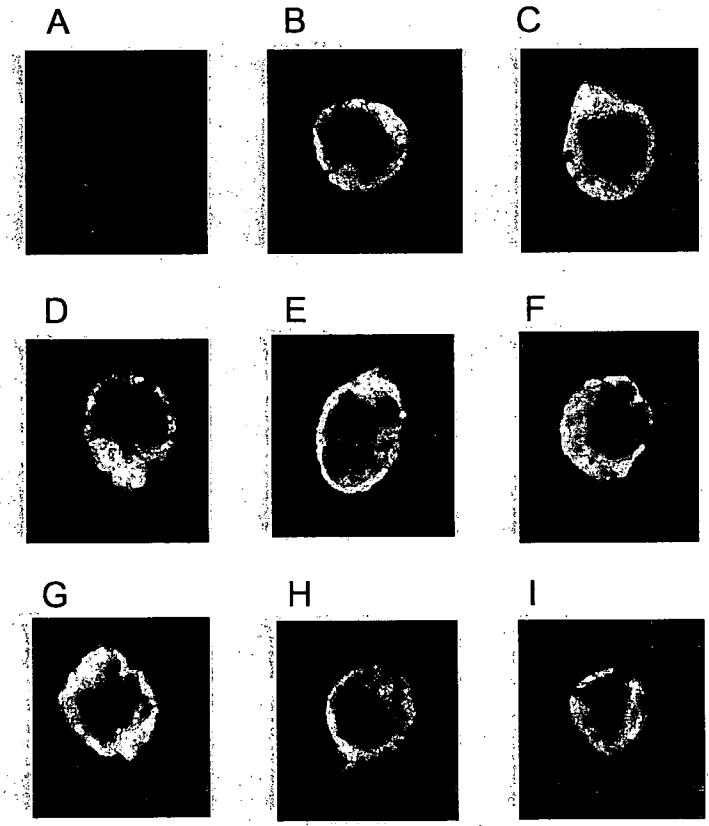

Fig. 6. Localization of FLAG-rMATE1 protein in HEK293 cells transiently transfected with vector alone (A), wild-type rMATE1 (B), or the H385Q (C), C62G (D), C126G (E), C129G (F), C356G (G), C392G (H), or C451G (I) mutant observed by confocal microscopy. HEK293 cells transfected with vector alone, wild-type cDNA, and mutant rMATE1 cDNA were fixed and stained with monoclonal anti-FLAG M2-FITC antibody.

of protein/min, $p < 0.05$; C126G: 8.68 ± 0.17 to 2.72 ± 0.09 pmol/mg of protein/min, $p < 0.05$, mean ± S.E., $n = 3$), indicating that cysteine residues other than Cys-62 and Cys-126 were sensitive to PCMBS. In contrast to wild-type rMATE1, the PCMBS-caused inhibition of [^{14}C]TEA uptake in these cysteine mutants was not protected by unlabeled TEA (C62G, 1.70 ± 0.19 pmol/mg of protein/min; C126G, 2.55 ± 0.41 pmol/mg of protein/min; mean ± S.E., $n = 3$). These results raised the possibility that at least both Cys-62 and Cys-126 contributed to the substrate binding.

pH Profile of TEA Uptake by rMATE1 H385Q. To investigate the role of the histidine residues, the pH profile of the uptake of [^{14}C]TEA by the rMATE1 H385Q mutant was examined. When the extracellular pH was changed from 6.0 to 8.5, the transport of [^{14}C]TEA by wild-type rMATE1 showed a bell-shaped curve with the greatest uptake value at pH 7.5. On the other hand, in the case of rMATE1 H385Q, no peak of the uptake was observed (Fig. 9).

Discussion

Recent molecular biological approaches have revealed that the mammalian MATE family functions as the renal H^+/organic cation antiport system. MATE1 can transport not only a typical organic cation TEA (Otsuka et al., 2005a; Ohta et al., 2006; Terada et al., 2006) but also various cationic drugs such as cimetidine and metformin and zwitter ion cephalexin (Terada et al., 2006). Furthermore, we recently found that hMATE1 also transports platinum agents (Yonezawa et al., 2006). To understand the molecular mechanisms behind the multispecificity of MATE1, it is necessary to define substrate-binding and/or recognition sites located in the transporter protein. Among amino acid residues, cysteine and histidine are of interest because we found previously that sulfhydryl groups and histidine residues are essential for the transport activity of H^+/organic cation antiport system using rat renal brush-border membrane vesicles (Hori et al., 1987, 1989) and a pig kidney epithelial cell line, LLC-PK$_1$ (Inui et al., 1985; Saito et al., 1992). Based on these backgrounds, in the present study, functional roles of cysteine and histidine residues of the MATE family, especially rMATE1, were examined.

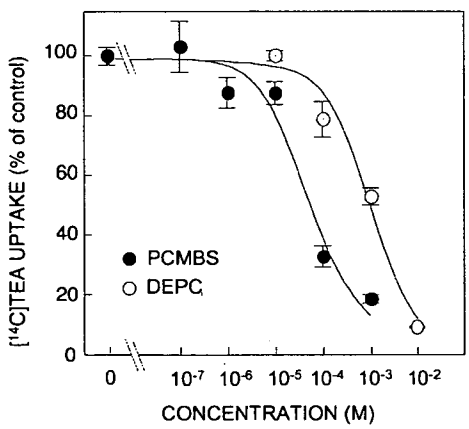

Fig. 7. Effects of DEPC (○) and PCMBS (●) on the uptake of TEA by rMATE1-expressing HEK293 cells. FLAG-rMATE1 cDNA was transfected into the HEK293 cells. The cells were preincubated at 25°C for 10 min with various concentrations of DEPC, pH 6.0, or PCMBS, pH 7.4. After incubation, the cells were rinsed twice with incubation medium and preincubated with incubation medium, pH 7.4, in the 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, pH 7.4, for 1 min at 37°C. Each point represents the mean ± S.E. of three monolayers. This figure is a representative one from three separate experiments.

By mutational analysis, we found that Cys-62 and Cys-126 of rMATE1, which are located in the first and the third transmembrane domain, respectively, played critical roles in the transport activity of TEA (Figs. 1 and 2). It is interesting that the corresponding cysteine residues of hMATE1 and hMATE2-K also function as essential amino acid residues (Fig. 4), suggesting that these cysteine residues play critical roles in the MATE family. Furthermore, protection by the substrate against PCMBS-caused inhibition of the transport of TEA via rMATE1 (Fig. 8) suggested that cysteine residues of rMATE1 function as substrate-binding sites. Protection assay using rMATE1 C62G and C126G mutants suggested that both Cys-62 and Cys-126 are involved in the substrate binding, although we cannot rule out the possibility that other cysteine residues participated in the substrate recognition. Pelis et al. (2006) have recently found that Cys-474 of hOCT2, which is suggested to be located in the 11th transmembrane helix that participates in the formation of the hydrophilic cleft, contributes to substrate-protein interaction. Because OCTs and

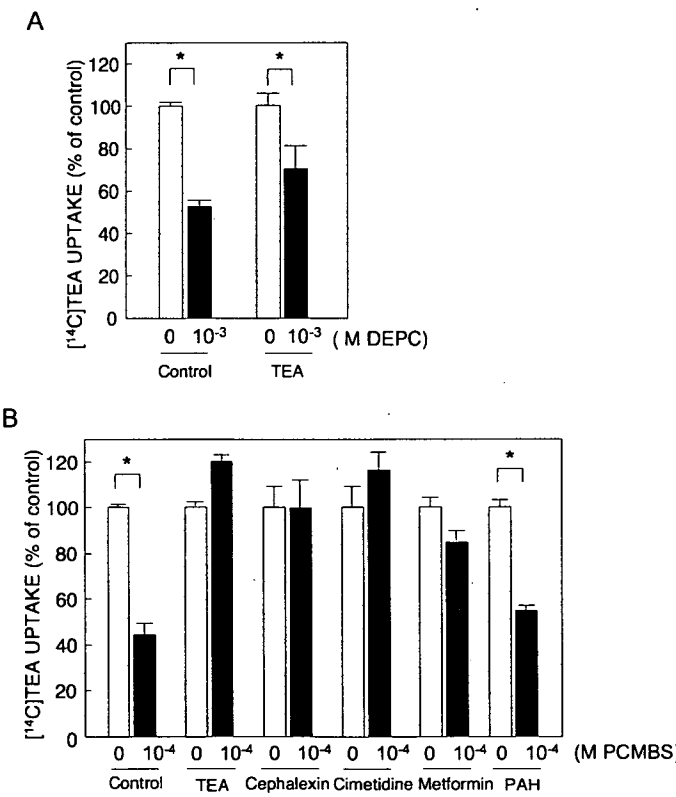

Fig. 8. Effects of unlabeled compounds on the inhibition of [^{14}C]TEA uptake by DEPC (A) or PCMBS (B)-pretreated rMATE1-expressing HEK293 cells. The cells were preincubated at 25°C for 10 min with or without unlabeled TEA (10 mM) and DEPC (1 mM), pH 6.0 (A) or were preincubated at 25°C for 10 min with or without unlabeled compounds (10 mM TEA, cephalexin, metformin, and PAH or 10 μM cimetidine) and PCMBS (0.1 mM) pH 7.4 (B). After the incubation, the cells were rinsed twice with incubation medium and preincubated with incubation medium, pH 7.4, in the 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, pH 7.4, for 1 min at 37°C. Each column represents the mean ± S.E. of three monolayers. This figure is a representative one from two separate experiments. *, $p < 0.05$, significantly different from wild type.

MATEs have similar substrate specificity, although their driving forces are quite different, it is reasonable that the same amino acid cysteine is involved in the substrate recognition. The present results strongly suggest that Cys-62 and Cys-126 of rMATE1 play an important role for substrate-interaction sites.

Most of the His-385 mutants of rMATE1 (Figs. 2 and 3) and corresponding histidine mutants of hMATE1 and hMATE2-K (Fig. 4) did not have the TEA transport activity. Furthermore, the histidine modifier reagent DEPC also inhibited the transport of TEA via rMATE1 (Fig. 7). In contrast to the effect of PCMBS, the DEPC-caused inhibition of TEA transport was not blocked in the presence of excess TEA (Fig. 8), suggesting that histidine residue of rMATE1 does not serve as substrate-binding site. In other H^+-coupled transporters such as H^+/peptide cotransporter 1 (Uchiyama et al., 2003) and Na^+/H^+ exchanger (Cha et al., 2003), histidine residues function as an H^+-binding site. It is, therefore, suggested that histidine residue of the MATE family acts as a H^+-binding site for driving force.

The H^+/organic cation antiport system is very sensitive to pH. The uptake of TEA was optimal at pH 7.0, and the uptake was markedly decreased at either an acidic or alkaline pH in renal brush-border membrane vesicle (Maegawa et al., 1988). No peak in the uptake of TEA by the rMATE1 H385Q mutant was observed when the pH of the medium changed gradually (Fig. 9). His-385 of rMATE1 may be important to the bell-shaped transport activity and function not only in making the driving force but as a regulator of substrate transport. Further studies such as detailed kinetic analyses may be needed. To our knowledge, there has been no report that a histidine residue is involved in the transport of a substrate by the MATE family.

Site-directed mutagenesis revealed that Asp-32, Glu-251, and Asp-367 of NorM protein, which is a member of the *Vibrio parahaemolyticus* MATE family, are essential for the Na^+-driven organic cation export (Otsuka et al., 2005b). Mutated hMATE1 with Glu-273 replaced with glutamine, the counterpart of Glu-251 of NorM protein, lacked TEA transport activity (Otsuka et al., 2005a). This glutamate residue is also conserved among rMATE1, mMATE1, and hMATE2-K. Previous studies using renal brush-border membrane vesicles treated with chemical modifiers revealed that carboxylate groups are critical for transport activity but are not involved in the substrate binding (Sokol et al., 1987). Alternatively, it was speculated that carboxylate groups are responsible for H^+ translocation process. It is therefore suggested that cysteine residues in the mammalian MATE family play a more important role for the substrate recognition than other amino acid residues. In MATE1, cysteine residues are located in the first and third transmembrane domains. The development of a three-dimensional model of MATE1 will clarify the molecular interaction of these amino acid residues with cationic substrates, as proposed for rat OCT1 (Popp et al., 2005) and rabbit OCT2 (Zhang et al., 2005).

In conclusion, we demonstrated that His-385, Cys-62, and Cys-126 in rMATE1 and corresponding amino acid residues of hMATE1 and hMATE2-K play an important role for the transport activity of MATE family. Cysteine residues of MATE1 make a key contribution to substrate recognition. This is the first study to identify the histidine and cysteine residues essential to the mammalian MATE family.

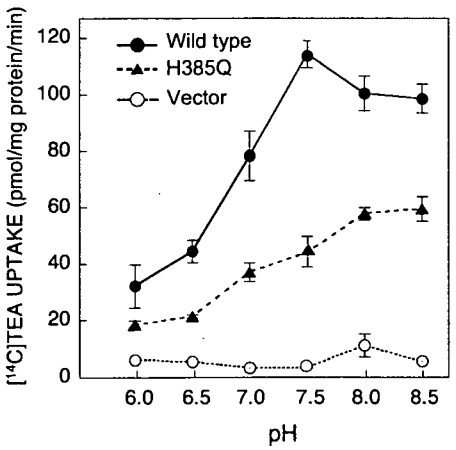

Fig. 9. Uptake of [^{14}C]TEA by HEK293 cells expressing wild-type rMATE1 (●), the H385Q mutant (▲), or vector alone (○). The cells were preincubated with incubation medium, pH 7.4, in the presence of 30 mM ammonium chloride for 20 min. Then the preincubation medium was removed, and the cells were incubated with 5 μM [^{14}C]TEA, (pH 6.0 to 8.5, for 1 min at 37°C. Each point represents the mean ± S.E. of three monolayers. This figure is a representative one from two separate experiments.

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Hepatocyte nuclear factor-4α regulates the human organic anion transporter 1 gene in the kidney

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Ogasawara K, Terada T, Asaka J, Katsura T, Inui K. Hepatocyte nuclear factor-4α regulates the human organic anion transporter 1 gene in the kidney. *Am J Physiol Renal Physiol* 292: F1819–F1826, 2007. First published March 6, 2007; doi:10.1152/ajprenal.00017.2007.—Human organic anion transporter 1 (OAT1, SLC22A6), which is localized to the basolateral membranes of renal tubular epithelial cells, plays a critical role in the excretion of anionic compounds. OAT1 is regulated by various pathophysiological conditions, but little is known about the molecular mechanisms regulating the expression of OAT1. In the present study, we investigated the transcriptional regulation of OAT1 and found that hepatocyte nuclear factor (HNF)-4α markedly transactivated the OAT1 promoter. A deletion analysis of the OAT1 promoter suggested that the regions spanning −1191 to −700 base pairs (bp) and −140 to −79 bp were essential for the transactivation by HNF-4α. These regions contained a direct repeat separated by two nucleotides (DR-2), which is one of the consensus sequences binding to HNF-4α, and an inverted repeat separated by eight nucleotides (IR-8), which was recently identified as a novel element for HNF-4α, respectively. An electrophoretic mobility shift assay showed that HNF-4α bound to DR-2 and IR-8 under the conditions of HNF-4α overexpression. Furthermore, under normal conditions, HNF-4α bound to IR-8, and a mutation in IR-8 markedly reduced the OAT1 promoter activity, indicating that HNF-4α regulates the basal transcription of OAT1 via IR-8. This paper reports the first characterization of the human OAT1 promoter and the first gene in the kidney whose promoter activity is regulated by HNF-4α.

SLC22A6; proximal tubule; promoter; inverted repeat-8

THE ORGANIC ANION TRANSPORTER (OAT) family plays an important role in the renal excretion of endogenous and exogenous organic anions, including drugs, toxins, and hormones (8, 18, 33, 41). Among the OAT family, OAT1 (SLC22A6) and OAT3 (SLC22A8) are localized to the basolateral membranes of the renal proximal tubular epithelial cells (26), and mRNA levels of both transporters were higher than those of other members of organic ion transporter (SLC22A) family in the human kidney cortex (42). So far, the molecular natures of OAT1 and OAT3 have been well characterized with regard to transport characteristics, structure-function relationships, and regulation (1, 8, 42, 47). As for regulation, various pathophysiological conditions, including hyperuricemia (14), renal failure (25, 36), bilateral ureteral obstruction (43), and acute biliary obstruction (6), affected the expression of OAT1 and/or OAT3. Although the elucidation of their regulatory mechanisms is quite important, studies that address the point are limited.

Recently, Kikuchi et al. (23) and we (28) reported the transcriptional regulation of the *OAT3* gene. In contrast, as for the OAT1 promoter, a computational analysis but not a functional analysis has been conducted (2). In the present study, therefore, we attempted to characterize the transcriptional regulation of OAT1 by focusing on two kinds of transcription factors: hepatocyte nuclear factor (HNF)-1α/β and HNF-4α. The former is reported to stimulate the promoter activity of OAT3 (23), while the latter transactivates the *OAT2* and *OCT1* genes, which are other members of the SLC22A family mainly expressed in the liver (31, 34).

HNF-1α/β are homeodomain-containing transcription factors that are expressed in the liver, kidney, intestine, stomach, and pancreas (3, 10). In the kidney, HNF-1α is reported to control the expression of transporters such as sodium/glucose cotransporter 2 and sodium/phosphate cotransporter 1 besides OAT3 (9, 30). Furthermore, Oat1 had markedly lower renal mRNA levels in Hnf-1α-null mice than in wild-type mice (24). On the other hand, HNF-4α is an orphan member of the nuclear receptor superfamily that is expressed in the liver, kidney, intestine, stomach, and pancreas (27, 40). Although few reports are available on the physiological role of HNF-4α in the kidney, HNF-4α protein has been found only in proximal tubular epithelial cells in the kidney (22), where OAT1 protein is located (26). Accordingly, it is possible that the *OAT1* gene is regulated by HNF-1α/β or HNF-4α. In the present study, we cloned the human OAT1 promoter region and examined whether the *OAT1* gene is regulated by these transcription factors.

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). An antibody against HNF-4 α (H-171) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The human HNF-1α and HNF-1β expression vectors were kindly supplied by Dr. Marco Pontoglio (Institute Pasteur, Paris, France). The human HNF-4α (transcript variant 2) expression vector was from OriGene Technologies (Rockville, MD).

5′-Rapid amplification of cDNA ends. To identify the transcription start site of human OAT1, 5′-rapid amplification of cDNA ends (5′-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 OAT1 (accession number NM_004790), 5′-GGTCCCACTCAGTCACGATGGTAGATGG-3′ (683 to 656); and a nested gene-specific primer for OAT1, 5′-CGACACCCCCCACCTGCTGCAGGAGG-3′ (347 to 322). 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 OAT1 gene. Based on the human genomic sequence (accession number NT_033903), the 2,747-

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Table 1. *Oligonucleotide sequence of primers*

Name	Sequence (5′-3′)	Position
	Primers for cloning of the OAT1 promoter	
OAT1/−2747*Nhe*I-F	GGGCTAGCAGTGGAGAAGGGTGTTAGAGCAGAC	−2747 to −2723
OAT1/+88*Xho*I-R	GGCTCGAGTCCCTTGCAGCTTCTCCTCACTTTG	+88 to +64
	Primers for the 5′-deleted constructs	
OAT1/−700*Nhe*I-F	CTGCTAGCGACACTATGGACAGAAGACAAT	−700 to −679
OAT1/−536*Nhe*I-F	CTGCTAGCATGGTTCTATAAGATGTGAGC	−536 to −516
OAT1/−270*Nhe*I-F	CTGCTAGCAAGGGCAGAAACTCCTCAAGA	−270 to −250
OAT1/−140*Nhe*I-F	CTGCTAGCTCTCCCTGCTCCTATTCAGTC	−140 to −120
OAT1/−79*Nhe*I-F	CTGCTAGCCTGACACAAGGAATCCTTGGA	−79 to −59
OAT1/−40*Nhe*I-F	CTGCTAGCCCAAGTCACACTTTAACTCATT	−40 to −19
OAT1/+88*Xho*I-R	GGCTCGAGTCCCTTGCAGCTTCTCCTCACTTTG	+88 to +64
	Primers for the site-directed mutagenesis	
OAT1/mutDR-2-B-F	GAGATTGCACCATTGCAAACCAGCCTGGGCAACAG	−880 to −846
OAT1/mutDR-2-B-R	CTGTTGCCCAGGCTGGTTTGCAATGGTGCAATCTC	−846 to −880
OAT1/mutIR-8-B-F	CTATTCAGTCCACCCTCTCCTGCTTTTTATAACCACTTGG	−129 to −90
OAT1/mutIR-8-B-R	CCAAGTGGTTATAAAAAGCAGGAGAGGGTGGACTGAATAG	−90 to −129
	Oligonucleotides for EMSA	
(−882/−853)-F	CTGAGATTGCACCATTGCACTCCAGCCTGG	−882 to −853
(−882/−853)-R	CCAGGCTGGAGTGCAATGGTGCAATCTCAG	−853 to −882
(−127/−100)-F	ATTCAGTCCACCCTCTCCTGCCCTTTAT	−127 to −100
(−127/−100)-R	ATAAAGGGCAGGAGAGGGTGGACTGAAT	−100 to −127
mutDR-2-A-F	CTGAGATTGCAAAATTGCACTCCAGCCTGG	−882 to −853
mutDR-2-A-R	CCAGGCTGGAGTGCAATTTTGCAATCTCAG	−853 to −882
mutDR-2-B-F	CTGAGATTGCACCATTGCAAACCAGCCTGG	−882 to −853
mutDR-2-B-R	CCAGGCTGGTTTGCAATGGTGCAATCTCAG	−853 to −882
mutDR-2-AB-F	CTGAGATTGCAAAATTGCAAACCAGCCTGG	−882 to −853
mutDR-2-AB-R	CCAGGCTGGTTTGCAATTTTGCAATCTCAG	−853 to −882
mutIR-8-A-F	ATTCTTTCCACCCTCTCCTGCCCTTTAT	−127 to −100
mutIR-8-A-R	ATAAAGGGCAGGAGAGGGTGGAAAGAAT	−100 to −127
mutIR-8-B-F	ATTCAGTCCACCCTCTCCTGCTTTTTAT	−127 to −100
mutIR-8-B-R	ATAAAAAGCAGGAGAGGGTGGACTGAAT	−100 to −127
mutIR-8-AB-F	ATTCTTTCCACCCTCTCCTGCTTTTTAT	−127 to −100
mutIR-8-AB-R	ATAAAAAGCAGGAGAGGGTGGAAAGAAT	−100 to −127

OAT1, organic anion transporter 1; F, forward; R, reverse; mut, mutant; DR, direct repeat; IR, inverted repeat. *Nhe*I and *Xho*I sites are underlined. Mutations introduced into the oligonucleotides are shown in bold.

base pair (bp) 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 the *Nhe*I and *Xho*I sites. This full-length reporter plasmid is hereafter referred to as −2,747/+88.

Preparation of deletion reporter constructs. The 5′-deleted constructs (−1851/+88, −1191/+88) were generated by digestion of the −2747/+88 construct with *Mlu*I and either *Nde*I or *Pst*I. The ends were blunted with T4 DNA polymerase (Takara Bio, Otsu, Japan) and then self-ligated. The other 5′-deleted constructs were generated by PCR with primers containing an *Nhe*I site and *Xho*I site (Table 1). The site-directed mutation in direct repeat (DR)-2 was introduced into the −2747/+88 construct and the mutation in inverted repeat (IR)-8 was introduced into the −2747/+88 construct and −140/+88 construct with a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the primers listed in Table 1. The nucleotide sequences of these deleted or mutated constructs were verified.

Cell culture, transfection, and luciferase assay. Opossum kidney (OK) cells were cultured in medium 199 (Invitrogen, Carlsbad, CA) containing 10% FBS (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 expression vector, and 25 ng of the *Renilla reniformis* vector pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation.

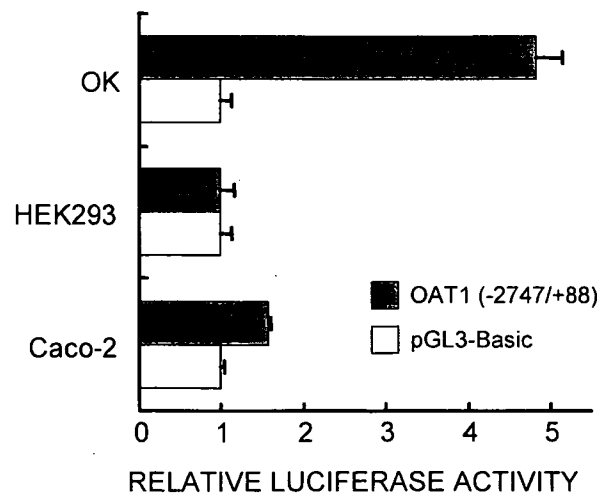

Fig. 1. Functional analysis of the human organic anion transporter 1 (OAT1) promoter in kidney-derived cell lines [opposum kidney (OK) and HEK293] and a human intestinal cell line (Caco-2). The longest OAT1 construct −2747/+88 (500 ng) was transfected into these cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative fold-increase compared with pGL3-Basic and represent the means ± SD of 3 replicates.