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-PK1. 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 (Ciaramboli et al., 2005; Yonezawa et al., 2006) and oxaliplatin (Yonezawa et al., 2006). Drug-drug interaction between cetirizine, a new histamine H1 blocker, and pillicainide, 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)
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 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-32P]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′-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 multipacillary 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/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, AstII, PsiI, 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 Jolla, CA).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotide sequences of primers</th>
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<tr>
<td>Name</td>
<td>Sequence</td>
</tr>
<tr>
<td>5′-RACE</td>
<td>Gene-specific primer</td>
</tr>
<tr>
<td></td>
<td>Nested gene-specific primer</td>
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<td>hOCT2</td>
<td>cloning</td>
</tr>
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<td>+23</td>
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<tr>
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</tr>
<tr>
<td>Reverse</td>
<td>5′-GATCCCTCTCGAGTTAGTGTGCTGACCTCTTTGATGACG-3′</td>
</tr>
<tr>
<td>EMSA probe</td>
<td>Collagen gene</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-AAGAGATTAACAACTACAGTACCGTCT-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-CTGGAATTCCTCCTCAACAGAGATGAGGGG-3′</td>
</tr>
<tr>
<td>Collagen mutCCAAT</td>
<td>Forward</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-AGAACCATGATGTTGCTAGATTTGCTCT-3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-GGCCTGTGCGCCAACATTTGCTTCT-3′</td>
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<tr>
<td>Reverse</td>
<td>5′-AGAAAACACAGCTGTTTGGCCCAAAAGGC-3′</td>
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<tr>
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<tr>
<td>Reverse</td>
<td>5′-AGAAAACACAGCTGTTTGGCCCAAAAGGC-3′</td>
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<tr>
<td>HO-1 mutE-box</td>
<td>Forward</td>
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<tr>
<td>Reverse</td>
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<tr>
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<tr>
<td>Reverse</td>
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</tr>
</tbody>
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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 pCNA3.1 (Invitrogen, Carlsbad, CA), and its sequence was verified.

Cell Culture and Luciferase Assay. The porcine kidney epithelial cell line LLC-PK1 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-PK1 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-32P]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-PK1 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 diethiothreitol, 10 mM NaF, 0.1 mM Na3VO4, 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 relatively 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 statistically 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 (Gorbolev 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-PK1 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-PK1 cells, and luciferase activity was measured (Fig. 1). The longest reporter construct (−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 (Ranji and Foka, 2002) and nuclear factor-Y (Mantovani, 1999), and the E-box is stimulated by USFs (Corre and Galiert, 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-PK1 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
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.

**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-PK1 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...
CAGGTG, 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 gene polymorphism, characterized by an extra TA repeat in the 5'-flanking region involved in the gene expression (Alves et al., 2001). In addition, it has been reported that a SNP of SLC22A5 (OCTN2) affects the transcription of OCTN2, contributing to the pathogenesis of Crohn's disease (Peltekova et al., 2004). A SNP within the E-box core motif also modulates gene regulation. For example, a single base transition within the USF 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.

References


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