Title

Regulation of Tissue-specific Expression of Renal Organic Anion Transporters by Hepatocyte Nuclear Factor 1 α/β and DNA Methylation

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Running title

Kidney-specific expression of organic anion transporters

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Abbreviations: OAT, organic anion transporter; URAT1, urate transporter 1; OATP, organic anion transporting polypeptide; HNF, hepatocyte nuclear factor; TSS, transcriptional start site; T-DMR, tissue-dependent differentially methylated region; h, human; m, mouse
Abstract

We have previously reported that the kidney- and liver-specific expression of transporters in mice involve the coordinated regulation by Hepatocyte Nuclear Factor (HNF) 1 and DNA methylation. The present study was aimed at investigating the role of this cascade in the transcriptional regulation of renal organic anion transporters yet to be characterized in human and mouse. Luciferase assays and electrophoretic mobility shift assays demonstrated that HNF1α/β enhances the promoter activity of Organic Anion Transporter (OAT) 4/SLC22A11 via binding to the HNF1-motif located nearby the transcriptional start site (TSS). DNA methylation profiles of human OAT1, OAT3, OAT4 and URAT1 were determined in human liver and kidney cortex by bisulfite sequencing. Most of the CpG dinucleotides around the TSSs of OAT1 and OAT3 were highly methylated in the liver compared with kidney cortex, being consistent with their tissue specificity, while the difference in the DNA methylation status was less remarkable between the two tissues for OAT4 and URAT1. Mouse Oat1 gene also contained CpG dinucleotides hypomethylated in the kidney and hypermethylated in the liver downstream its TSS, whereas two of the seven CpG dinucleotides around the TSS of mouse Oat3 were significantly methylated in the liver compared with the kidney. Taken together, these findings underscored the central role of HNF1α/β in the transcriptional regulation of OATs and highlighted DNA methylation-dependent gene silencing as one of the mechanisms underlying the tissue-specific transactivation by this master regulator.
Introduction

Organic Anion Transporter (OAT) 1/SLC22A6, OAT3/SLC22A8 and Urate Transporter 1 (URAT1)/SLC22A12 are predominantly expressed in the kidney, whereas OAT4/SLC22A11 is expressed in the kidney and placenta (Sekine et al., 2006; Giacomini et al., 2010; VanWert et al., 2010). OAT1 and OAT3 mediate the basolateral uptake of anionic drugs and their metabolites from the systemic circulation (Hasegawa et al., 2003; Nozaki et al., 2007; El-Sheikh et al., 2008). Meanwhile, OAT4 and URAT1 are expressed on the brush border membrane of renal proximal tubule epithelial cells (Enomoto et al., 2002; Ekaratanawong et al., 2004). URAT1 is specifically involved in the renal reabsorption of urate (Enomoto et al., 2002), while OAT4, like OAT1 and OAT3, is characterized by its broad substrate specificity, and considered to mediate tubular secretion and/or reabsorption process in the kidney (Cha et al., 2000; Ekaratanawong et al., 2004).

Several groups including us have investigated the transcriptional regulation of OATs which underlies their predominant expression in the kidney. The promoters of OAT1, OAT3, and URAT1 are directly transactivated by Hepatocyte Nuclear Factor (HNF) 1α and/or HNF1β, two isoforms of HNF1, with less transactivation potency of HNF1β in both human and mouse (Kikuchi et al., 2006; Kikuchi et al., 2007; Saji et al., 2008). In accordance with these in vitro results, Hnf1α-null mice showed marked reduction in the mRNA expression of Oat1 and Urat1 and moderate reduction in that of Oat3 compared with wild-type mice (Maher et al., 2006; Kikuchi et al., 2007). In silico analyses also located two tandem HNF1-motifs in the putative promoter region of human OAT4, from −97 to −85 and from −41 to −29 (designated as HNF1-motif1 and HNF1-motif2, respectively) relative to the transcriptional start site (TSS) (Fig. 3).
and their physiological significance has yet to be established.

HNF1α is also involved in the constitutive expression of liver-specific organic anion transporters such as Organic Anion Transporting Polypeptide (OATP) 1B1 and OATP1B3 in human and Oatp1b2 in mouse (Jung et al., 2001). This highlights the discrepancy in the tissue distribution between HNF1α/β and its downstream transporters. We have previously demonstrated that DNA methylation-dependent gene silencing underlies the kidney-specific expression of human OAT3 and mouse Urat1 (Kikuchi et al., 2006; Kikuchi et al., 2007). Subsequently, a genome-wide DNA methylation analysis together with bioinformatics approaches located tissue-dependent differentially methylated regions (T-DMRs) that are hypomethylated in the liver but hypermethylated in the extrahepatic tissues in a number of liver-specific genes including transporters, and many of those genes contained HNF1-binding sequences in their promoters (Yagi et al., 2008; Imai et al., 2009). Most recently, we also identified T-DMRs in the kidney-specific amino acid transporters that are potentially transactivated by Hnf1α/β (Kikuchi et al., 2010). These observations highlighted the fundamental role of the regulatory cascade comprising HNF1 and DNA methylation in the tissue-specific transporter expression. Multiple CpG dinucleotides are also localized around the TSS of h/mOAT1, h/mOat3, hOAT4, and hURAT1 (Fig. 3), and it remains to be examined whether they serve as T-DMRs.

The present study was aimed at elucidating the involvement of HNF1 and DNA methylation in the transcriptional regulation of renal organic anion transporters yet to be characterized, namely OAT1, OAT3, OAT4, and URAT1 in human, and Oat1 and Oat3 in mouse.
Material and Methods

Materials

All reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

Isolation of the 5'-Flanking Region of Human OAT4 Gene

The TSS of the human OAT4 gene was identified using the public database, Database of Transcriptional Start Sites (http://dbtss.hgc.jp/), with reference sequence identification of OAT4 (NM_018484). DNA fragments of varying length from the 5'-flanking region of OAT4 gene were amplified by PCR using human genomic DNA as a template and the following primer sets: forward, −826, −534, or −244, and reverse +17 (Supplemental Table). The primers were designed according to the sequence of the 5'-flanking region of OAT4 gene, and the number indicates the position of primers relative to the TSS (+1). The forward and reverse primers contained an artificial KpnI and BglII site, respectively. The resulting PCR products (−826/+17, −534/+17, and −244/+17) were digested with KpnI and BglII after subcloning into pGEM-T Easy vector (Promega, Madison, WI) and ligated into pGL3-Basic vector (Promega) predigested with KpnI and BglII, yielding the following promoter constructs: −826/+17-Luc, −534/+17-Luc, and −244/+17-Luc. The −47/+17-Luc and −26/+17-Luc constructs were generated by in vitro annealing of sense and antisense oligonucleotides corresponding to each region (Supplemental Table), which was followed by ligation into pGL3-Basic vector predigested with KpnI and BglII. The sequence identity of all promoter constructs with respective genomic sequences was verified by DNA sequencing. Plasmid DNA was prepared using the GenElute Plasmid Midiprep kit (Sigma-Aldrich, St. Louis, MO).
Site-Directed Mutagenesis

Mutations in tandem HNF1-motifs located within the human OAT4 putative promoter region were introduced using QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with internally mutated oligonucleotides (Supplemental Table) according to the manufacturer’s instructions. The introduction of mutations was verified by DNA sequencing. The positions and bases that replaced the original sequences were decided based on the information described in the database of transcription factors TRANSFAC (http://www.gene-regulation.com/); highly conserved bases in the consensus HNF1-motif were mutated into bases with the least frequency at the corresponding position.

Cell Culture, Transfections, and Luciferase Assays

Cell culture, transfections, and luciferase assays were performed as described previously (Kikuchi et al., 2006). Briefly, 0.5 μg of each promoter construct and 0.05 μg of internal standard pRL-SV40 were transfected into HepG2 or Caco-2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For transactivation assays in HEK293 cells, 0.5 μg empty pcDNA3.1(+) vector, 0.5 μg HNF1α expression vector, 0.25 μg HNF1α and HNF1β expression vectors, or 0.5 μg HNF1β expression vector was cotransfected with 0.5 μg of the corresponding promoter construct and 0.05 μg of pRL-SV40 using FuGENE6 (Roche Diagnostics, Indianapolis, IN). The promoter activity was measured as relative light units of Firefly luciferase per unit of Renilla luciferase.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from HEK293 cells transiently transfected with pcDNA3.1(+), either HNF1α or HNF1β expression vector, or both expression vectors
as described previously (Kikuchi et al., 2007). Three kinds of double-stranded oligonucleotides were generated by hybridizing single-stranded complementary oligonucleotide with sense sequences shown in Supplemental Table. The sequence “wt”, “per”, and “mut” correspond to the wild-type distal HNF1-motif found in the OAT4 promoter from −97 to −85 (HNF1-motif1), the perfect consensus sequence for the HNF1-motif, and the wild-type sequence mutated in the HNF1-motif which is the same mutation as used in the luciferase assays, respectively. Five micrograms of nuclear extracts from HEK293 cells transfected with several expression vectors were used in the electrophoretic mobility shift assays. Competition and supershift assays were performed as described previously with Dig Gel Shift Kit, 2nd Generation (Roche Diagnostics) (Kikuchi et al., 2006).

Sodium Bisulfite Genomic Sequencing

Human tissue specimens were obtained from non-profit organization Human and Animal Bridging Research Organization (Tokyo, Japan). Genomic DNA from three individual specimens of human liver and kidney cortex, and liver and kidney of C57BL/6NCrj male mouse at 11 weeks of age was extracted using a Get Pure DNA Kit (Dojindo Molecular Technologies, Gaithersburg, MD). One or 2 micrograms of genomic DNA digested with EcoRI (human) or BamHI (mouse) were subjected to bisulfite reaction as described previously (Kikuchi et al., 2007). The DNA fragments covering the proximal promoter region of human OAT1, OAT3, OAT4, and URAT1 genes, and mouse Oat1 and Oat3 genes were amplified by PCR using the primers shown in Supplemental Table. PCR was performed using Immolace DNA polymerase (Bioline, London, UK) under the following conditions: 94 °C for 10 min; 43 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min; and final extension of
72 °C for 10 min. The PCR products were cloned into pGEM-T Easy vector (Promega), and 10 or 11 clones were chosen randomly from each sample and sequenced to determine the presence of methylated cytosines. For human OAT1, OAT3, OAT4, and URAT1, the degree of methylation was calculated at each CpG dinucleotide in the three individual liver and kidney cortex specimens, and these values were statistically compared between the two tissues using Student’s t-test. For mouse Oat1 and Oat3, differences in the methylation status of each CpG dinucleotide between the liver and kidney were statistically analyzed using the Quantification tool for Methylation Analysis (QUMA; http://quma.cdb.riken.jp/) with Fisher’s exact test. Significant differences between the two tissues were denoted by an asterisk (*, P < 0.05).
Results

Transactivation of the human OAT4 promoter by HNF1α/β.

Computational analysis using MatInspector mapped two tandem HNF1-motifs within 100 bp upstream the TSS of OAT4: one from −97 to −85 (HNF1-motif1) and the other from −41 to −29 (HNF1-motif2). HEK293 cells lack the endogenous expression of HNF1α and HNF1β, making this cell line suitable for investigating the effect of exogenously expressed HNF1α and HNF1β on the transcriptional activity (Kikuchi et al., 2006). A series of 5′-truncated promoter constructs of OAT4, with or without mutations in the HNF1-motif1 and/or HNF1-motif2, were transfected into HEK293 cells together with HNF1α and/or HNF1β expression vectors (Fig. 1A). Exogenous expression of HNF1α and/or HNF1β dramatically increased the luciferase activity of the wild-type OAT4 promoter constructs containing both HNF1-motifs (−826/+17-Luc, −534/+17-Luc, and −244/+17-Luc) compared with the pcDNA3.1(+) transfected control. The luciferase activity of −26/+17-Luc, which contained neither HNF1-motif1 nor HNF1-motif2, was unaffected by exogenously expressed HNF1α and/or HNF1β, while that of −47/+17-Luc, which contained only HNF1-motif2, was slightly enhanced. Mutations in either HNF1-motif1 or HNF1-motif2 (−244/+17_m1-Luc or −244/+17_m2-Luc) partially attenuated the luciferase activity caused by the forced-expression of HNF1α and/or HNF1β, and mutations in both motifs (−244/+17_m3-Luc) completely abolished the luciferase activity. These results indicate that the transcription of OAT4 is stimulated by HNF1α/β through both the HNF1-motif1 and motif2 when the amount of HNF1α/β is in excess rather than physiologically relevant.

HepG2 and Caco-2 cells exhibit endogenous expression of HNF1α alone and
both HNF1\(\alpha\) and HNF1\(\beta\) at the protein level, respectively (Kikuchi et al., 2006). In both cell lines, slight and dramatic increase was observed in the luciferase activity when extending the promoter region from \(-26\) to \(-47\) and from \(-47\) to \(-244\), respectively, compared with promoter-less pGL3-basic vector (Fig. 1B). Further increase was not remarked by extending the promoter region up to \(-826\) bp. Mutations in the HNF1-motif1 alone (\(-244/+17\)\_m1-Luc) or both motifs (\(-244/+17\)\_m3-Luc) significantly reduced the promoter activity, whereas those in HNF1-motif2 alone (\(-244/+17\)\_m2-Luc) did not alter the activity of wild-type construct. These results suggest the predominant role of HNF1-motif1 located from \(-97\) to \(-85\) in the transcriptional activity of \(OAT4\) in the presence of physiological level of HNF1\(\alpha/\beta\).

Direct binding of HNF1\(\alpha/\beta\) to the \(OAT4\) promoter.

Electrophoretic mobility shift assays were performed to examine the interaction of HNF1\(\alpha\) and/or HNF1\(\beta\) with the HNF1-motif1 in the promoter region of \(OAT4\). Nuclear extract from HEK293 cells transiently transfected with pcDNA3.1(+), either HNF1\(\alpha\) or HNF1\(\beta\) expression vector, or both expression vectors was incubated with the \(wt\) probe in the presence or absence of \(per\) or \(mut\) competitor (Fig. 2A). A non-specific band, the formation of which was abolished by both \(per\) and \(mut\) competitors, was observed when the probe was incubated with nuclear extract (lanes 2-11). One band (band \(a\) or \(b\)) was formed with nuclear extract from HEK293 cells transfected with either HNF1\(\alpha\) or HNF1\(\beta\), respectively (lanes 3 and 9). In addition to the band \(a\), a faint signal was observed which migrated faster than the band \(a\) but slower than the band \(b\) with nuclear extracts from the cells transfected with both expression vectors, while the band \(b\) was not detected in the same sample (lane 6).
The formation of bands $a$ and $b$ as well as the slight signal between them were abolished by 25-fold molar excess of unlabeled per competitor (lanes 4, 7, and 10), but not by mut competitor (lanes 5, 8 and 11). No specific shifted band was observed when the probe was incubated with nuclear extract from HEK293 cells transfected with pcDNA3.1(+) (lane 2).

Supershift assays were performed to confirm the specificity of the binding using a specific antibody against HNF1$\alpha$ or HNF1$\beta$ (Fig. 2B). The addition of an antibody against HNF1$\alpha$ supershifted the band $a$ (lane 4 and 7) but not band $b$ (lane 10), while the addition of an antibody against HNF1$\beta$ supershifted the band $b$ (lane 11) but not band $a$ (lanes 5 and 8). These results suggest that the bands $a$ and $b$ correspond to the binding of HNF1$\alpha$/HNF1$\alpha$ homodimer and HNF1$\beta$/HNF1$\beta$ homodimer to the HNF1-motif1 in the $OAT4$ promoter, respectively. The slight signal between the bands $a$ and $b$ observed in lane 6 was abolished by the addition of either antibodies, indicating that this signal may represent the binding of HNF1$\alpha$/HNF1$\beta$ heterodimer.

The interaction of HNF1$\alpha$/HNF1$\alpha$ or HNF1$\beta$/HNF1$\beta$ homodimer with the HNF1-motif2 was much weaker than that with the HNF1-motif1, almost barely detectable, in the same experimental condition (data not shown).

**DNA methylation profiles of human renal organic anion transporters.**

We searched the location of CpG dinucleotides from 500 bp upstream to 250 bp downstream of the TSS for human $OAT1$, $OAT3$, $OAT4$, and $URAT1$ genes (Fig. 3). CpG dinucleotides were found only in the downstream region of the TSS for $OAT1$, while there were multiple CpG dinucleotides surrounding the TSS of $OAT3$, $OAT4$, and $URAT1$. The methylation status of selected CpG dinucleotides in these regions was determined by bisulfite genomic sequencing in human liver and kidney cortex from
three individuals based on the availability of PCR primers suitable for the experiments.

Then, the degree of methylation at each CpG dinucleotide was calculated for individual tissue specimen, and statistically analyzed between the two tissues. All the CpG dinucleotides downstream the TSS of OAT1 were significantly methylated in the liver albeit the presence of interindividual differences to some extent; the CpG dinucleotides at +196 and +205 are highly methylated in Liver-1 compared with Liver-2 and -3 (Fig. 3A). The degree of methylation was significantly higher in the liver than in the kidney cortex at 11 out of 16 CpG dinucleotides around the TSS of OAT3, wherein it was low/moderate at −155 and −148 in all the liver specimens, moderate at −263, −254, and −84 in Liver-2, and low/moderate at −241 and −84 in Liver-3 (Fig. 3B). A significant difference in the DNA methylation status was observed in four CpG dinucleotides located at −475, −459, −429, and −336 relative to the TSS of OAT4; those were relatively highly methylated in the liver especially in Liver-1 but almost unmethylated in the kidney cortex (Fig. 3C). The CpG dinucleotides at −210, −208, +131, and +150 were methylated to variable degree across individuals both in the liver and kidney cortex, and the difference didn’t achieve statistical significance. On the other hand, there was a large interindividual differences in the DNA methylation of URAT1 promoter, where only the one at −181 was significantly hypermethylated in the liver compared with the kidney cortex (Fig. 3D).

DNA methylation profiles of mouse renal organic anion transporters.

The DNA methylation profiles in the genomic region surrounding the functional HNF1-motifs of mouse Oat1 and Oat3 were investigated in the liver and kidney. For Oat1, the difference in the DNA methylation status was prevalent in the downstream of
TSS; five CpG dinucleotides located at +4, +13, +82, +140, and +148 were hypomethylated in the kidney but hypermethylated in the liver whereas those at −523, −467, and −454 were hypermethylated in both tissues (Fig. 4A). Meanwhile, two of the seven CpG dinucleotides around the TSS of *Oat3*, located at −396 and +58, were significantly hypomethylated in the kidney compared with the liver (Fig. 4B). The degree of methylation was high at −573 and −481, and low to moderate at −279, −251, and −86 in both tissues.
Discussion

In the present study, we examined the hypothesis that the kidney-specific expression of organic anion transporters is achieved by a common mechanism, which is, the coordinated regulation comprising the transcriptional activation by HNF1α/β and repression by DNA methylation.

The regulatory mechanism of the mRNA expression of human OAT4 was characterized. In silico analysis revealed the presence of tandem HNF1-motifs in the promoter region of OAT4, which was unique among the organic anion transporters within SLC22A family. Luciferase assays and electrophoretic mobility shift assays demonstrated the involvement of HNF1α and/or HNF1β in the minimal promoter activity of OAT4, with the distal HNF1-motif playing a dominant role in the interaction with these transcription factors (Figs. 1 and 2). In the kidney, HNF1 normally exists as HNF1α/HNF1β heterodimer or HNF1β/HNF1β homodimer in the proximal tubules, whereas HNF1β/HNF1β homodimer predominates in the other segments (Lazzaro et al., 1992; Pontoglio et al., 1996). Since the expression of OAT4 is restricted to proximal tubules (Ekaratanawong et al., 2004) and HNF1α/HNF1β heterodimer is more potent than HNF1β/HNF1β homodimer in the transactivation of OAT4 promoter (Fig. 1A), it is most likely that HNF1α/HNF1β heterodimer is the master regulator for the constitutive expression of OAT4 in the kidney cortex. This agrees with what has been observed for OAT1, OAT3, and URAT1 in both human and mouse (Kikuchi et al., 2006; Kikuchi et al., 2007; Saji et al., 2008).

DNA methylation status of OATs and URAT1 were compared between human liver and kidney cortex. Liver was selected as a counterpart based on the negligible expression of OATs and URAT1 regardless of the strong expression of HNF1α/β.
The CpG dinucleotides downstream the TSS of *OAT1* and those around the TSS of *OAT3* were relatively methylated in the liver compared with the kidney cortex, although there were some interindividual variations in the degree of methylation in the liver depending on the location (Fig. 3A and B). These methylation profiles coincided with the kidney-specific expression of both *OAT1* and *OAT3* (Hosoyamada et al., 1999; Cha et al., 2001). There was a sharp contrast in the number of differentially methylated CpG dinucleotides between transporters. Unlike *OAT1* and *OAT3*, only four out of 14 and 1 out of 13 CpG dinucleotides around the TSS of *OAT4* and *URAT1* were significantly hypomethylated in the kidney cortex compared to the liver, respectively (Fig. 3C and D). The functional relevance of each CpG dinucleotide to the transcriptional suppression needs further investigation.

There were marked interindividual differences in the methylation status of *OAT4* and *URAT1* promoters in the kidney cortex; CpG dinucleotides at −210, −208, +131, and +150 were methylated in the kidney cortex-1 for *OAT4*, and almost all CpG dinucleotides were moderately methylated in the kidney cortex-2 for *URAT1*, when compared to the other two individuals (Fig. 3C and D). We speculate that this leads to the interindividual differences in the mRNA expression and potentially alteration in the transport activity of *OAT4* and *URAT1*. *URAT1* is responsible for the reabsorption of urate in the kidney, and non-synonymous mutations in the *URAT1* gene, causing functional impairment, are associated with idiopathic renal hypouricemia (Anzai et al., 2007). Aberrant DNA methylation could be a new mechanism affecting the transcription of *URAT1*, and thereby causing hypouricemia in certain individuals. Further studies are necessary to elucidate the relevance of the epigenetic regulation in the interindividual variation of *OAT4* and *URAT1* expression in human kidney.
It is of great interest to see whether the epigenetic signatures are conserved for organic anion transporters across species. Combined with our previous literature about mouse Urat1, we have comprehensively analyzed the DNA methylation profiles of the homologous kidney-specific transporters in human and mouse except for OAT4, whose mouse counterpart is yet to be determined or missing. The location of T-DMR was similar between human and mouse for OAT1; the CpG dinucleotides downstream of the TSS were hypomethylated in the kidney while hypermethylated in the liver in both species (Figs. 3A and 4A). It has been demonstrated that T-DMR is retained across species for sphingosine kinase-1 gene; a CpG island is mapped adjacent to 5’-end of the gene in human, rat, and mouse, and the T-DMR at the 5’ edge of the CpG island is conserved between rat and mouse, suggesting the indispensable role of this region in the tissue-specific expression (Imamura et al., 2004). Thus, the similar localization of T-DMR relative to the TSS of OAT1/Oat1 indicates the functional relevance of this region to the kidney-specific expression of these homologous genes. On the other hand, T-DMR was pronounced in human rather than mouse for OAT3 (Figs. 3B and 4B), and vice versa for URAT1 (Kikuchi et al., 2007) by unknown reason. This indicates the different contribution of epigenetic system to the transcriptional regulation between mice and human, and might explain the different tissue distribution of these transporters among species; human OAT3 and URAT1 are predominantly expressed in the kidney cortex (Cha et al., 2001; Enomoto et al., 2002) while mouse Oat3 and Urat1 are expressed at the endothelial cells of brain capillaries and epithelial cells of choroid plexus in addition to the kidney cortex (Imaoka et al., 2004; Ohtsuki et al., 2004).

OAT1 and OAT3, and OAT4 and URAT1 genes occur in the genome as tightly
linked pair on the same chromosome (Chr 11) with intergenic distances; 8.3 kbp for $OAT1$ and $OAT3$, and 19.9 kbp for $OAT4$ and $URAT1$ in human (Eraly et al., 2003). Both gene pairs are transcribed in the same direction with $OAT3$ and $OAT4$ upstream of $OAT1$ and $URAT1$, respectively. Gene duplication from the same ancestral gene might have resulted in the variety of organic anion transporters with different substrate specificities, while they maintained the common ancestral regulatory system for their transcription which facilitates almost the same tissue distribution of each gene pair. Taking into account their proximity in the genome, it is also possible that DNA methylation in the promoter region of $OAT3$ and $OAT4$ may induce the condensed structure of chromatin throughout the widespread genomic region including the $OAT1$ and $URAT1$ loci, respectively, and inactivate the transcription of neighboring genes besides their corresponding genes.

In conclusion, the present study has highlighted the conserved mechanism underlying the kidney-specific expression of organic anion transporters in human and mouse. The minimal promoter activities of these transporters are highly dependent on the transactivation by HNF1α and/or HNF1β. In addition, the release from DNA methylation-dependent gene silencing may underlie the tissue-selective transactivation by HNF1 of at least some organic anion transporters in the kidney.
Authorship Contributions

Participated in research design: Kikuchi, Kusuhara and Sugiyama

Conducted experiments: Jin, Saji and Kikuchi

Performed data analysis: Kikuchi, and Kusuhara

Wrote or contributed to the writing of the manuscript: Kikuchi, Kusuhara and Sugiyama
References


Mouse reduced in osteosclerosis transporter functions as an organic anion transporter 3 and is localized at abluminal membrane of blood-brain barrier. *J Pharmacol Exp Ther* **309**:1273-1281.


Footnote

L.J. and R.K. contributed equally to this work.

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Legends for figures

Figure 1  Transactivation of \( OAT4 \) promoter by HNF1\( \alpha \) and HNF1\( \beta \).

(A) Exogenous expression of HNF1\( \alpha \) and/or HNF1\( \beta \) in HEK293. HEK293 cells were transfected with a series of 5’-truncated wild-type promoter constructs of \( OAT4 \), promoter constructs with mutations in the HNF1-motif1 and/or HNF1-motif2, or a promoterless pGL3-Basic plasmid, together with empty pcDNA3.1(+) vector (white bars), HNF1\( \alpha \) expression vector (black bars), HNF1\( \alpha \) and HNF1\( \beta \) expression vectors (gray bars), or HNF1\( \beta \) expression vector (hatched bars). The luciferase activity was measured as described in Materials and Methods and was shown as the factor of induction over background activity measured in cells transfected with pGL3-Basic together with pcDNA3.1(+).  (B) Analysis of \( OAT4 \) promoter function in HepG2 and Caco-2. HepG2 (white bars) and Caco-2 cells (gray bars) were transiently transfected with a series of 5’-truncated wild-type promoter constructs of \( OAT4 \), promoter constructs with mutations in the HNF1-motif1 and/or HNF1-motif2, or a promoterless pGL3-Basic plasmid. The luciferase activity was measured as described in Materials and Methods and was shown as the factor of induction over background activity measured in cells transfected with pGL3-Basic in each cell line. All results are presented as the mean ± S.E. of triplicate samples. Representative graphs of at least two independent experiments are shown.

Figure 2  HNF1\( \alpha \) and HNF1\( \beta \) directly bind to \( OAT4 \) promoter.

(A) Competition assays. A digoxigenin-labeled probe corresponding to the \( OAT4 \) wild-type promoter containing the distal HNF1-motif (HNF1-motif1) was incubated with nuclear extracts of HEK293 cells transfected with HNF1\( \alpha \) and/or HNF1\( \beta \), or
pcDNA3.1(+) as a negative control, in the presence or absence of a 25-fold excess of unlabeled competitor (per or mut) as indicated. (B) Supershift assays. The probe was incubated with nuclear extracts of HEK293 cells transfected with HNF1α and/or HNF1β in the presence or absence of a specific antibody against HNF1α (α) or HNF1β (β) as indicated. The DNA-protein complex was detected as described in Materials and Methods.

**Figure 3** DNA methylation profiles of OAT1, OAT3, OAT4, and URAT1 in human.

(A) Schematic diagram of the genomic region around the TSS. The vertical lines and numbers indicate the positions of cytosine residues of CpGs relative to the TSS (+1). The positions of the HNF1 motifs are shown by rectangles. (B) Bisulfite genomic sequencing was performed to determine the DNA methylation profiles of OAT1, OAT3, OAT4, and URAT1 with genomic DNA extracted from three individual specimens of human liver and kidney cortex as described in Materials and Methods. DNA methylation status of individual CpGs. The open and closed circles represent unmethylated and methylated cytosines, respectively. The positions of the most upstream and downstream CpGs in the region analyzed are given in the figure. *P < 0.05, a significant difference between the two tissues.

**Figure 4** DNA methylation profiles of Oat1 and Oat3 in mouse.

Bisulfite genomic sequencing was performed to determine the DNA methylation profiles of Oat1 (A) and Oat3 (B) with genomic DNA extracted from liver and kidney of male mouse as described in Materials and Methods. Top, a schematic diagram of
the genomic region around the TSS. The vertical lines and numbers indicate the positions of cytosine residues of CpGs relative to the TSS (+1). The positions of the HNF1 motifs are shown by rectangles. Bottom, DNA methylation status of individual CpGs. The open and closed circles represent unmethylated and methylated cytosines, respectively. *P < 0.05, a significant difference between the two tissues.
Fig. 1 (A)
Fig. 1 (B)
Fig. 2 (A)

Nuclear extract

<table>
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<th>Competitor</th>
<th>-</th>
<th>pcDNA3.1(+)</th>
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<th>HNF1α/HNF1β</th>
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<td>a</td>
<td></td>
<td>per</td>
<td>mut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td>per</td>
<td>mut</td>
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Non-specific band

Free probe

Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11
---|---|---|---|---|---|---|---|---|---|----|---
Fig. 2 (B)

The figure shows a gel analysis with lanes labeled 1 to 11. The lanes are divided into four groups:

1. **Nuclear extract**
2. **Antibody**
3. **Supershifted bands**
4. **Non-specific band**
5. **Free probe**
6. **- pcDNA3.1(+)**
7. **HNF1α**
8. **HNF1α/HNF1β**
9. **HNF1β**

Each group has lanes labeled 1 to 11, with specific markers indicating the presence or absence of proteins or antibodies.

Lane 1 shows a band labeled 'a', Lane 2 shows a band labeled 'b', and Lane 3 shows a band labeled 'α'. Lane 4 shows a band labeled 'β'. The remaining lanes show variations of these markers, indicating different combinations of proteins and antibodies.
<table>
<thead>
<tr>
<th></th>
<th>Liver-1</th>
<th>Liver-2</th>
<th>Liver-3</th>
<th>Kidney cortex-1</th>
<th>Kidney cortex-2</th>
<th>Kidney cortex-3</th>
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<td>hURAT1</td>
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Fig. 4A

mOat1

mOat3

TSS (+1)

HNF1

HNF1

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Fig. 4 (B)

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