

# Regulation of the Expression of Human Organic Anion Transporter 3 by Hepatocyte Nuclear Factor 1 $\alpha$ / $\beta$ and DNA Methylation<sup>[S]</sup>

Ryota Kikuchi, Hiroyuki Kusuhara, Naka Hattori, Kunio Shiota, Insook Kim, Frank J. Gonzalez, and Yuichi Sugiyama

*Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences (R.K., H.K., Y.S.), and Laboratory of Cellular Biochemistry, Department of Animal Resource Sciences/Veterinary Medical Sciences (N.H., K.S.), University of Tokyo, Tokyo, Japan; and Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland (I.K., F.J.G.)*

Received April 8, 2006; accepted June 20, 2006

## ABSTRACT

Human organic anion transporter 3 (hOAT3/SLC22A8) is predominantly expressed in the proximal tubules of the kidney and plays a major role in the urinary excretion of a variety of organic anions. The promoter region of hOAT3 was characterized to elucidate the mechanism underlying the tissue-specific expression of hOAT3. The minimal promoter of hOAT3 was identified to be located approximately 300 base pairs upstream of the transcriptional start site, where there are canonical TATA and hepatocyte nuclear factor (HNF1) binding motifs, which are conserved in the rodent Oat3 genes. Transactivation assays revealed that HNF1 $\alpha$  and HNF1 $\beta$  markedly increased hOAT3 promoter activity, where the transactivation potency of HNF1 $\beta$  was lower than that of HNF1 $\alpha$ . Mutations in the HNF1 binding motif prevented the transactivation. Electrophoretic mobility shift assays demonstrated binding of the HNF1 $\alpha$ /HNF1 $\alpha$  ho-

modimer or HNF1 $\alpha$ /HNF1 $\beta$  heterodimer to the hOAT3 promoter. It was also demonstrated that the promoter activity of hOAT3 is repressed by DNA methylation. Moreover, the expression of hOAT3 was activated de novo by forced expression of HNF1 $\alpha$  alone or both HNF1 $\alpha$  and HNF1 $\beta$  together with the concomitant DNA demethylation in human embryonic kidney 293 cells that lack expression of endogenous HNF1 $\alpha$  and HNF1 $\beta$ , whereas forced expression of HNF1 $\beta$  alone could not activate the expression of hOAT3. This suggests a synergistic action of the HNF1 $\alpha$ /HNF1 $\alpha$  homodimer or HNF1 $\alpha$ /HNF1 $\beta$  heterodimer and DNA demethylation for the constitutive expression of hOAT3. These results indicate that the tissue-specific expression of hOAT3 might be regulated by the concerted effect of genetic (HNF1 $\alpha$  and HNF1 $\beta$ ) and epigenetic (DNA methylation) factors.

Tubular secretion plays a significant role in the urinary excretion of many compounds together with filtration in the glomeruli. Cumulative evidence suggests that organic anion transporter 1 (OAT1/SLC22A6) and organic anion transporter 3 (OAT3/SLC22A8) are predominantly involved in the tubular secretion of anionic drugs and drug metabolites as well as endobiotics at the basolateral membrane of the kidney proximal tubules (Van Aubel et al., 2000; Hasegawa et

al., 2002, 2003; Robertson and Rankin, 2005; Sekine et al., 2006). Generation of Oat1 and Oat3 null mice confirmed an essential role for these transporters in the renal transport (Sweet et al., 2002; Eraly et al., 2006). In contrast to the functional characterization of these transporters, the regulatory mechanism of the basal expression of OAT1 and OAT3 remains to be elucidated.

In the present study, we focused on hepatocyte nuclear factor 1 (HNF1), consisting of two isoforms, HNF1 $\alpha$  and HNF1 $\beta$ , as a key regulator of OAT3 expression. HNF1 is a homeodomain-containing factor that is expressed in the epithelia of a variety of organs, including liver, kidney, intestine, stomach, and pancreas (Blumenfeld et al., 1991; Mendel and Crabtree, 1991; Tronche and Yaniv, 1992). In the kidney, expression of HNF1 $\alpha$  is confined to the proximal tubules,

This work was supported by a Health and Labor Sciences research grant from the Ministry of Health, Labor and Welfare for the Research on Advanced Medical Technology.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.106.025494.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

**ABBREVIATIONS:** OAT, organic anion transporter; HNF1, hepatocyte nuclear factor 1; h, human; PCR, polymerase chain reaction; m, mouse; EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; DTT, dithiothreitol; 5azadC, 5-aza-2'-deoxycytidine; wt, wild-type HNF1 sequence; per, perfect consensus sequence; mut, mutated consensus sequence; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBS-T, Tris-buffered saline/0.05% Tween 20; nt, nucleotide(s).

whereas that of HNF1 $\beta$  is observed along the tubular epithelial cells throughout the entire nephron (Lazzaro et al., 1992; Pontoglio et al., 1996). HNF1 is functionally composed of three domains: an N-terminal dimerization domain, a homeobox-like DNA-binding domain, and a C-terminal transactivation domain. Both the dimerization domain and the DNA-binding domain of HNF1 $\alpha$  and HNF1 $\beta$  show high homologies, enabling them to form heterodimers as well as homodimers and to recognize the same DNA sequences (Mendel et al., 1991). In contrast, their transactivation domains are more divergent, and HNF1 $\alpha$  is a more potent transactivator than HNF1 $\beta$  (Rey-Campos et al., 1991).

HNF1 $\alpha$  is involved in the regulation of a number of hepatic genes, including albumin,  $\alpha$ 1-antitrypsin, and  $\alpha$ - and  $\beta$ -fibrinogen (Mendel and Crabtree, 1991; Tronche and Yaniv, 1992), and also some of the transporters expressed predominantly in liver (Jung et al., 2001; Shih et al., 2001). The importance of HNF1 $\alpha$  in kidney proximal tubules has become apparent after studies using two lines of HNF1 $\alpha$ -null mice as well as conventional in vitro studies (Pontoglio et al., 1996; Lee et al., 1998). One line of HNF1 $\alpha$ -null mice suffers from renal Fanconi syndrome, a defect in renal proximal tubule reabsorption, leading to glucosuria, aminoaciduria, phosphaturia, and polyuria. Indeed, HNF1 $\alpha$  plays an essential role in the expression of sodium glucose cotransporter 2 and sodium/phosphate cotransporter 1, which are involved in the reabsorption of filtered glucose and phosphate from the urine, respectively (Pontoglio et al., 2000; Cheret et al., 2002).

In contrast to HNF1 $\alpha$ , the role of HNF1 $\beta$  in adult animals remains unclear, because the homologous inactivation of the HNF1 $\beta$  gene results in embryonic lethality at embryonic day 7.5 as a result of a defect in visceral endoderm differentiation (Barbacci et al., 1999; Coffinier et al., 1999). However, more recently, HNF1 $\beta$  was shown to be essential for the formation of a functional bile duct system and several hepatic metabolic functions, by means of the conditional gene-targeting technique (Coffinier et al., 2002). HNF1 $\beta$  is also involved in the regulation of kidney-specific Ksp-cadherin promoter (Bai et al., 2002), and kidney-specific inactivation of HNF1 $\beta$  leads to a renal polycystic phenotype (Gresh et al., 2004).

Because multiple CpG dinucleotides, primary targets of DNA methylation in the vertebrate genome, are located in the putative promoter region of hOAT3 (Fig. 1), we also focused on the role of DNA methylation in regulation of hOAT3 expression. DNA methylation is one of the mechanisms underlying the epigenetic control of gene expression (Bird, 2002). Methylation of the CpG dinucleotides in the promoter region can evoke the condensed structure of chromatin in the neighboring region through the recruitment of chromatin remodeling factors, such as methyl CpG binding proteins and histone deacetylases, which prevents transactivation by most of the transcription factors. Therefore, there is an inverse correlation between gene expression and DNA methylation in the promoter region. During the last decade, the role of DNA methylation in mammalian embryogenesis, differentiation, and progression of cancer has been highlighted. More recently, DNA methylation has been recognized to regulate the tissue-specific expression of many genes (Shiota, 2004). Whether these epigenetic mechanisms are involved in the regulation of transporter genes has remained largely unknown.

In the present study, we report the isolation of the hOAT3 promoter and demonstrate the involvement of HNF1 $\alpha$  and HNF1 $\beta$  in basal transcriptional activity. In addition, these studies revealed that DNA methylation is involved in the gene suppression of hOAT3, and transcriptional activation of hOAT3 by HNF1 $\alpha$  and HNF1 $\beta$  requires concomitant DNA demethylation of the promoter.

## Materials and Methods

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

**Isolation of the 5'-Flanking Region of the hOAT3 and mOat3 Gene.** The transcriptional start site of the hOAT3 gene was identified using the public database Database of Transcriptional Start Sites (<http://dbtss.hgc.jp/>), with the ref sequence identification for hOAT3 (GenBank accession number NM\_004254). DNA fragments of varying length from the 5'-flanking region of the hOAT3 gene were generated by polymerase chain reaction (PCR) using human genomic DNA as a template and the following primer sets:



**Fig. 1.** Multiple alignment of the putative proximal promoter of human, mouse, and rat OAT3. Nucleotide sequences of the 5'-flanking region of human (top), mouse (middle), and rat (bottom) OAT3 genes were aligned using Genetyx-Mac version 10 (Genetyx, Tokyo, Japan), so that the maximal homology of sequences among species could be obtained. Nucleotide numberings are relative to the transcriptional start sites (nt +1, arrow). Homologous sequences among species are boxed. Representative consensus binding motifs for putative regulatory elements are shaded with the respective transcription factors given above the sequence, and CpG dinucleotides in each sequence are reverse-colored.

forward, -1471, -644, or -308, and reverse +6 (Table 1). The primers were designed according to the sequence of the 5'-flanking region of the hOAT3 gene, and the number indicates the position of the primers relative to the transcriptional start site. The forward primers contained an artificial KpnI site and the reverse primers contained an artificial HindIII site. The resulting PCR products (-1471/+6, -644/+6, and -308/+6) were digested with KpnI and HindIII after subcloning into pGEM-T Easy vector (Promega, Madison, WI) and ligated into pGL3-Basic vector (Promega) predigested with KpnI and HindIII, yielding the following promoter constructs: -1471/+6-Luc, -644/+6-Luc, and -308/+6-Luc. The -35/+6-Luc construct was generated by in vitro annealing of the sense (-35/+6) and antisense (+6/-35) oligonucleotides (Table 1) followed by ligation into the pGL3-Basic vector that had been predigested with KpnI and HindIII. The mOat3 promoter fragment was PCR-amplified from mouse genomic DNA using the following primers: forward (-156) and reverse (+6) (Table 1). The amplified product was ligated into the pGL3-Basic vector as in the case of hOAT3, yielding the mOat3-Luc promoter construct. The sequence identity of all the constructs with the respective genomic sequences was verified by DNA sequencing. Plasmid DNA was prepared using the GenElute Plasmid Midiprep kit (Sigma-Aldrich, St. Louis, MO).

**Plasmid Constructions.** To generate HNF1 $\alpha$  and HNF1 $\beta$  expression vectors, the coding regions of human HNF1 $\alpha$  and HNF1 $\beta$  were amplified by PCR using primers containing HindIII and EcoRI restriction sites and inserted into the same sites of pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). The sequences of primers used for the amplification of HNF1 $\alpha$  and HNF1 $\beta$  are listed in Table 1. The entire sequences were verified by DNA sequencing.

**Site-Directed Mutagenesis.** The mutated promoter fragment (-308/+6-HNF1mut) having a 4-base pair disrupted HNF1 motif was generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) using internal mutated oligonucleo-

tides (Table 1). The introduction of the mutations was verified by DNA sequencing.

**In Vitro Methylation of Plasmid DNA.** Reporter constructs of the hOAT3 promoter were methylated in vitro with 3 U of SssI methylase (New England Biolabs, Beverly, MA) for each microgram of DNA in the presence of 160  $\mu$ M S-adenosylmethionine at 37°C for 3 h. Completion of the methylation was confirmed by resistance to HpaII digestion (data not shown).

**Cell Culture and Transfections.** HepG2 and Caco-2 cell lines were maintained in a culture medium consisting of Dulbecco's modified Eagle's medium with 4500 mg/l glucose (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), nonessential amino acids, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Invitrogen). The HEK293 cell line was maintained in a culture medium consisting of Dulbecco's modified Eagle's medium with 1000 mg/l glucose supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were seeded in 24-well culture plates ( $0.5 \times 10^5$  cells/well) 1 day before transfection. HepG2 and Caco-2 cells were transfected with 0.5  $\mu$ g of the corresponding promoter construct and 0.05  $\mu$ g of the internal standard pRL-SV40 vector (Promega) to normalize the transfection efficiency using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HEK293 cells were transfected with 0.5  $\mu$ g of the corresponding promoter and 0.05  $\mu$ g of pRL-SV40 using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). For cotransfection assays with HEK293 cells, 0 to 0.5  $\mu$ g of HNF1 $\alpha$  and HNF1 $\beta$  expression vectors or empty pcDNA3.1(+) vector was added to the transfection reaction. Then, 48 h after transfection, cells were lysed with passive lysis buffer, and the luciferase activities were assayed using the dual-luciferase reporter system (Promega) and quantified in a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). The promoter activity was measured as the relative light units of firefly luciferase per unit of *Renilla reniformis* luciferase.

TABLE 1

Oligonucleotides used for the production of promoter fragments, plasmid construction, mobility shift assays, site-directed mutagenesis, RT-PCR, and quantitative PCR

Regarding the oligonucleotides used for the mobility shift assays and site-directed mutagenesis, the HNF1 recognition motif in the hOAT3 promoter region is underlined. Bold type indicates the difference in the sequence of the per and mut compared with wt sequence found in the hOAT3 promoter.

| Oligonucleotide  | Orientation | Sequence (5' to 3')                                  |
|--|-------------|--|
| Primers and oligonucleotides used for cloning of 5'-flanking regions                             |             |  |
| hOAT3  |             |  |
| -1471  | Forward     | CGGGGTACCGGAACAGAGAGGTAAGGC                          |
| -644   | Forward     | CGGGGTACCGAGAGAAGCCTGTCCATTG                         |
| -308   | Forward     | CGGGGTACCGATTCTCTCCAGAACTCTCC                        |
| +6   | Reverse     | CCCAAGCTTCAAGCTGTGTTTGTGCCTCC                        |
| -35/+6   | Sense       | CCCTTATATAAGCCCCCTGGGGAGGCACAAACACAGCTTGA            |
| +6/-35   | Antisense   | AGCTTCAAGCTGTGTTTGTGCCTCCCCAGGGGGCTTATATAAGGGGTAC    |
| mOat3  |             |  |
| -156   | Forward     | CGGGGTACCATCAACAGCCTTGGCTGAGG                        |
| +6   | Reverse     | CCCAAGCTTCAAGCTGTGTTGTGTCTCC                         |
| Primers used for cloning of HNF1 $\alpha$ and HNF1 $\beta$                                       |             |  |
| HNF1 $\alpha$  | Forward     | AAGCTTGCCATGGTTTCTAAACTGAGCC                         |
|  | Reverse     | GAATTCGTGTTACTGGGAGGAAGAGGCC                         |
| HNF1 $\beta$   | Forward     | AAGCTTGAAAATGGTGTCCAAGCTCAGC                         |
|  | Reverse     | GAATTCGGCATCACCAGGCTTGTAGAGG                         |
| Oligonucleotides used for construction of EMSA probe and competitor or site-directed mutagenesis |             |  |
| wt   | Sense       | CGCAAAGAAAGTCAAAACATTAGCCCGGAAACAGC                  |
| per  | Sense       | CGCAAAGAAAGT <b>TAAT</b> CATT <b>AA</b> CCCGGAAACAGC |
| mut  | Sense       | CGCAAAGAAAGT <b>CACACATCGCC</b> CCCGGAAACAGC         |
| Primers used for RT-PCR or quantitative PCR  |             |  |
| hOAT3  | Forward     | GGCAGGTATACTGATTGGAG                                 |
|  | Reverse     | TCCACAGGATGATAGGAAG                                  |
| HNF1 $\alpha$  | Forward     | TGGGTCTACGTTACCAAC                                   |
|  | Reverse     | TCTGCACAGGTGGCATGAGC                                 |
| HNF1 $\beta$   | Forward     | TGCACAAAGCCTCAACACCT                                 |
|  | Reverse     | GTTGGTGAGTGTACTGATGC                                 |
| GAPDH  | Forward     | AATGACCCCTTCATTGAC                                   |
|  | Reverse     | TCCACGACGTACTCAGCGC                                  |



**Preparation of Nuclear Extracts.** Nuclear extracts were prepared from  $1.0 \times 10^7$  of HepG2, Caco-2, and HEK293 cells. Cell centrifugation and the subsequent steps to recover the nuclear proteins were all performed at 4°C. Cells were scraped off the plates, suspended in 0.5 ml of phosphate-buffered saline, and centrifuged at 1500g for 5 min. The cellular pellet was resuspended in 150  $\mu$ l of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.4% Nonidet P-40, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail; Sigma-Aldrich). After a 10-min incubation on ice, cells were centrifuged at 4°C and 3000 rpm for 5 min. The pellet was resuspended in 150  $\mu$ l of buffer A and centrifuged at 4°C and 3000 rpm for 5 min, and this process was repeated twice. The supernatant was removed, and the nuclear pellet was resuspended in 200  $\mu$ l of buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail) and incubated on ice for 30 min. The tube was centrifuged at 4°C and 10,000 rpm for 20 min, and the supernatant was recovered as the nuclear extract. Protein concentrations were measured by the method of Lowry et al. (1951).

**Electrophoretic Mobility Shift Assay.** The double-stranded oligonucleotide probes were obtained by hybridizing single-stranded complementary oligonucleotides with sense sequences as shown in Table 1. Digoxigenin-11-ddUTP was incorporated into each 3' end using a Dig Gel Shift kit, 2nd Generation (Roche Diagnostics). Sequence wt corresponds to the wild-type HNF1 sequence found in the hOAT3 promoter, and per corresponds to the perfect consensus sequence for HNF1, whereas mut denotes the wild-type sequence mutated within the HNF1 recognition motif. For EMSA, 5  $\mu$ g of nuclear extracts from HepG2, Caco-2, and HEK293 cells was incubated at room temperature for 15 min with 30 fmol of digoxigenin-labeled probe, 2  $\mu$ g of poly(dI-dC), and 0.1  $\mu$ g of poly-L-lysine in 20  $\mu$ l of 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1 mM DTT, 2% (w/v) Tween 20, and 30 mM KCl. For competition assays, a 25-fold excess of unlabeled dimerized oligonucleotides was added. For supershift experiments, 1  $\mu$ g of antibody against HNF1 $\alpha$  or HNF1 $\beta$  (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the reaction mixture. Reactions were analyzed by electrophoresis through Novex 6% DNA retardation gels (Invitrogen). After electrotransfer to a positively charged nylon membrane (Roche Diagnostics), bands were detected nonisotopically with a Dig Gel Shift kit, 2nd Generation, according to the manufacturer's instructions.

**5-Aza-2'-deoxycytidine Treatment and Analysis of hOAT3 Expression by Reverse Transcription-PCR.** Before the treatment with 5-aza-2'-deoxycytidine (5azadC) (DNA methylation inhibitor; Sigma-Aldrich), HepG2, Caco-2, and HEK293 cells were precultured for 24 h and then cultured for 72 h in medium containing 0, 1, 10, or 100  $\mu$ M 5azadC. To examine the synergetic effect of the expression of HNF1 $\alpha$  or HNF1 $\beta$  and DNA demethylation, HEK293 cells were plated in 24-well plates ( $0.5 \times 10^5$  cells/well) 1 day before transient transfection of 0 to 0.5  $\mu$ g of HNF1 $\alpha$  expression vector, HNF1 $\beta$  expression vector, or empty pcDNA3.1(+) vector. The transfection was performed using FuGENE 6 according to the manufacturer's instructions. After 12 h, the cells were treated with 0 or 100  $\mu$ M 5azadC, cultured for 48 h, and then subjected to RNA isolation. Total RNA was prepared from cells by a single-step guanidium thiocyanate procedure using ISOGEN (Nippon Gene, Toyama, Japan). The RNA was then reverse-transcribed using a random-nonamer primer (Takara, Shiga, Japan). PCR was performed with the forward and reverse primers listed in Table 1 to detect the partial fragments of hOAT3, HNF1 $\alpha$ , HNF1 $\beta$ , and GAPDH cDNA. PCR was performed under the following conditions: 94°C for 2 min; 40 cycles for hOAT3, 30 cycles for HNF1 $\alpha$  and HNF1 $\beta$ , and 25 cycles for GAPDH of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; final extension 72°C for 5 min.

**Quantitative PCR.** To quantify the mRNA expression of HNF1 $\alpha$  and HNF1 $\beta$  in HepG2, Caco-2, and HEK293 cells, real-time quantitative PCR was performed using a LightCycler and the appropriate software (version 3.53; Roche Diagnostics) according to the manu-

facturer's instructions. cDNA used for the quantification was prepared as described above. Primers for HNF1 $\alpha$  and HNF1 $\beta$  used in this study are shown in Table 1.

PCR was performed using a SYBR Premix Ex Taq (perfect real time) (Takara). The protocol for PCR was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, 55°C for 10 s, and 72°C for 15 s. A standard curve was generated by dilutions of the target PCR product, which had been purified and had its concentration measured. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis. The mRNA expression of HNF1 $\alpha$  and HNF1 $\beta$  in each cell line was normalized by the mRNA expression of GAPDH.

**Western Blotting.** Nuclear extracts prepared from HepG2 or Caco-2 cells were subjected to Western blot analysis to confirm the expression of HNF1 $\alpha$  and HNF1 $\beta$  at protein levels. In this, 30  $\mu$ g of nuclear proteins was electrophoresed on 10% SDS-polyacrylamide gel with a 4.4% stacking gel. Separated proteins were transferred to a polyvinylidene difluoride membrane using a blotter at 15 V for 1 h. The membrane was blocked with Tris-buffered saline/0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature and subsequently incubated with an antibody against HNF1 $\alpha$  or HNF1 $\beta$  (1:2000) at 4°C overnight. After washing three times with TBS-T for 5 min, the membrane was allowed to bind a horseradish peroxidase-labeled donkey anti-goat IgG (Chemicon International, Temecula, CA) diluted 1:5000 in TBS-T for 1 h at room temperature and detected using ECL Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

## Results

**Computational Analysis of the Potential Transcription Factor Binding Sites in hOAT3, mOat3, and rOat3 Minimal Promoters.** Sequence homologies to known gene regulatory elements in the hOAT3 5'-flanking region up to approximately nt -300 relative to the transcription start site were identified by a computer-based approach using Mat Inspector (<http://www.genomatix.de/>) (Fig. 1). Several potential transcription factor recognition sites were found in this region, including a TATA motif at nt -32 to -27, an HNF1 binding motif at nt -65 to -53, a cAMP response element-binding protein binding motif at nt -87 to -80, and a signal transducer and activator of transcription 5 binding motif at nt -302 to -294. In addition, there are 13 CpG dinucleotides in this region, which may be potential DNA methylation sites (Fig. 1). A homologous sequence to the hOAT3 5'-flanking region in the mouse and rat Oat3 genomic locus was obtained from the National Center for Biotechnology Information genome database. The transcriptional start site of mOat3 and rOat3 gene was suggested based on the high homology (approximately 70%) to the hOAT3 5'-flanking region (Fig. 1). The TATA motif and HNF1 motif were also found in the mOat3 and rOat3 putative promoters.

**Analysis of Basal hOAT3 Gene Promoter Activity.** To determine the minimal region of the hOAT3 proximal promoter required for its promoter activity, a series of deleted promoter constructs (-1471/+6-Luc, -644/+6-Luc, -308/+6-Luc, and -35/+6-Luc) were transfected into three kinds of human-derived cell lines (HepG2, Caco-2, and HEK293), and the luciferase activity in each cell line was measured (Fig. 2). The transfection of -1471/+6, -644/+6, and -308/+6 constructs resulted in increased luciferase activity compared with the promoterless pGL3-Basic plasmid in all cell lines, whereas no significant luciferase activity was observed after transfection of the -35/+6 construct. These

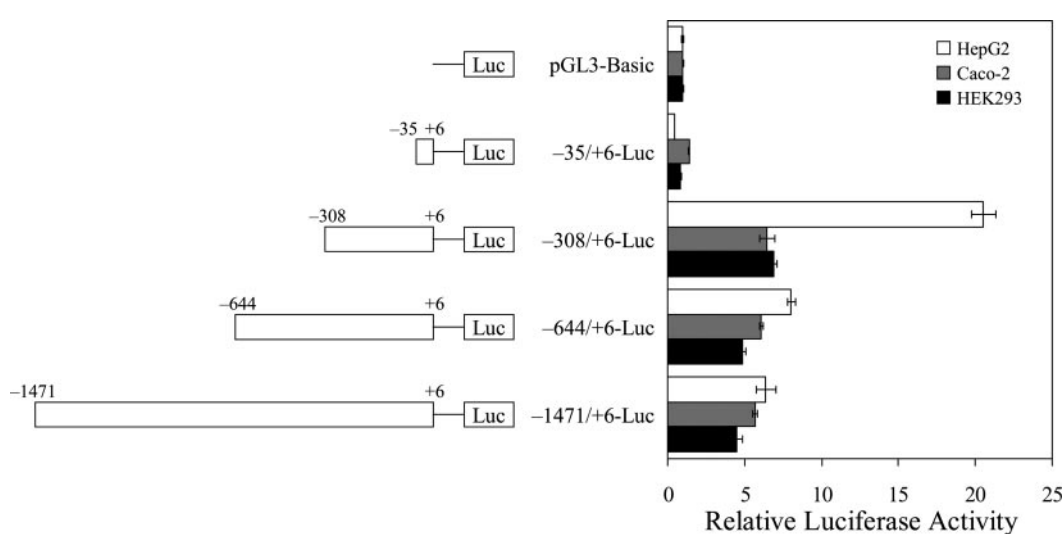
results suggest that  $-308$  to  $+6$  of the hOAT3 5'-flanking region can act as a minimal promoter. The luciferase activity of the  $-644/+6$  construct was much lower than that of the  $-308/+6$  construct only in HepG2 cells, suggesting the existence of some negative regulatory elements between  $-644$  and  $-308$  in this liver-derived tumor cell line.

**Expression Level of HNF1 $\alpha$  and HNF1 $\beta$  in HepG2, Caco-2, and HEK293 Cells.** Real-time quantitative PCR revealed that the expression level of HNF1 $\beta$  in HepG2 cells was 27% that of HNF1 $\alpha$  (Fig. 3A). The levels of HNF1 $\alpha$  and HNF1 $\beta$  mRNA in Caco-2 cells were 73 and 55% that of HNF1 $\alpha$  in HepG2 cells, respectively. In contrast, neither HNF1 $\alpha$  nor HNF1 $\beta$  was detected in HEK293 cells. Western blot analysis with nuclear extracts from HepG2 or Caco-2 cells demonstrated that in HepG2 cells, HNF1 $\alpha$  is predominantly expressed at the protein level, whereas in Caco-2 cells, both HNF1 $\alpha$  and HNF1 $\beta$  are expressed (Fig. 3B).

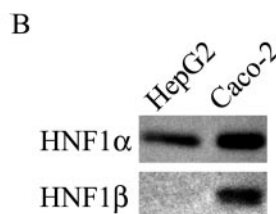
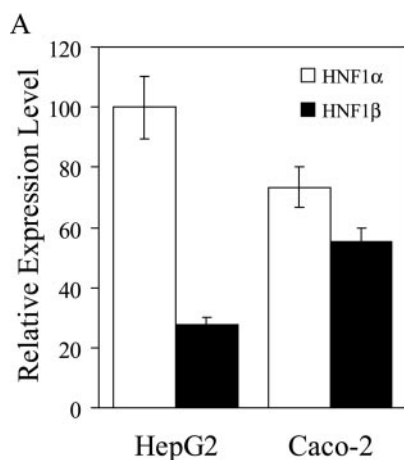
**Mutagenesis of the HNF1 Binding Motif.** To investigate the functional importance of the HNF1 binding motif in the hOAT3 promoter region for the basal promoter activity, mutations in this motif were introduced into the  $-308/+6$ -Luc construct, and the reporter activity was measured after transfection into HepG2, Caco-2, and HEK293 cells (Fig. 4). Mutations in the HNF1 binding motif attenuated the luciferase activity by approximately 50% compared with the wild-

type construct in HepG2 and Caco-2 cells where endogenous HNF1 $\alpha$  and HNF1 $\beta$  were detected by real-time quantitative PCR. In contrast, HNF1 motif disruption by site-directed mutagenesis had no effect on the transcriptional activity in HNF1 $\alpha$ - and HNF1 $\beta$ -deficient HEK293 cells. These results confirm that the HNF1 binding motif in the hOAT3 promoter is functional.

**Transactivation of the Promoter Activity by Exogenous Expression of HNF1 $\alpha$  and/or HNF1 $\beta$ .** The effect of exogenously expressed HNF1 $\alpha$  and HNF1 $\beta$  on the hOAT3 promoter activity was investigated by cotransfection assays in HEK293 cells. As shown in Fig. 5, independent or simultaneous transfection of HNF1 $\alpha$  and HNF1 $\beta$  enhanced the luciferase activity of the hOAT3  $-308/+6$  wild-type reporter construct ( $-308/+6$ -HNF1wt) compared with the pcDNA3.1(+)-transfected control. The luciferase activities of the promoterless pGL3-Basic or hOAT3  $-308/+6$  HNF1-mutated reporter construct ( $-308/+6$ -HNF1mut) were not stimulated by transfection of the HNF1 $\alpha$  and/or HNF1 $\beta$  expression vectors. These results provide clear evidence that both HNF1 $\alpha$  and HNF1 $\beta$  can transactivate the basal promoter activity of hOAT3 and that the effect is mediated by the intact HNF1 binding motif in the hOAT3 promoter. The luciferase activity of the wild-type reporter construct with the independent transfection of HNF1 $\alpha$  is higher than that



**Fig. 2.** Analysis of hOAT3 promoter function. HepG2 (white bars), Caco-2 (gray bars), and HEK293 cells (black bars) were transiently transfected with a series of deleted promoter constructs  $-35/+6$ -Luc,  $-308/+6$ -Luc,  $-644/+6$ -Luc, and  $-1471/+6$ -Luc, or a promoterless pGL3-Basic plasmid as described under *Materials and Methods*. Transfection efficiency was normalized by cotransfection of internal standard pRL-SV40. The promoter activity was measured as described under *Materials and Methods* and was shown as the factor of induction of luciferase over background activity measured in cells transfected with pGL3-Basic in each cell line. Results are presented as the mean  $\pm$  S.E. of triplicate samples.

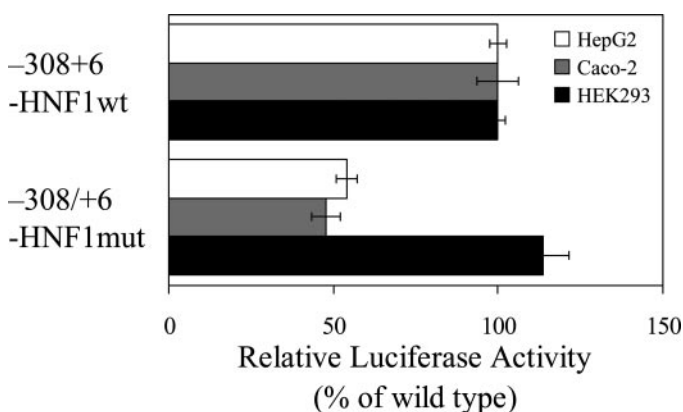


**Fig. 3.** Expression of HNF1 $\alpha$  and HNF1 $\beta$  in HepG2, Caco-2, and HEK293 cells. A, mRNA expression of HNF1 $\alpha$  and HNF1 $\beta$  was measured by real-time quantitative PCR using specific primers (Table 1), and the data were normalized by the mRNA expression of GAPDH. The relative expression level of HNF1 $\alpha$  (white columns) and HNF1 $\beta$  (black columns) in each cell line was given as a ratio with respect to the mRNA expression of HNF1 $\alpha$  in HepG2 cells which was taken as 100%. Results are presented as the mean  $\pm$  S.E. of three samples. B, Western blotting was performed to investigate the protein expression of HNF1 $\alpha$  and HNF1 $\beta$ . Nuclear extracts from HepG2 or Caco-2 cells were separated by SDS-polyacrylamide gel electrophoresis (10% separating gel), and HNF1 $\alpha$  and HNF1 $\beta$  were detected as described under *Materials and Methods*.

with the independent transfection of HNF1 $\beta$ , indicating that the transactivation potency of HNF1 $\beta$  is lower than that of HNF1 $\alpha$ . Simultaneous transfection of HNF1 $\alpha$  and HNF1 $\beta$  yielded intermediate luciferase activity, which may be explained by the formation of a HNF1 $\alpha$ /HNF1 $\beta$  heterodimer that enhances the promoter activity to the lesser degree than the HNF1 $\alpha$ /HNF1 $\alpha$  homodimer.

**HNF1 Binds to the hOAT3 Promoter.** EMSA was performed using the oligonucleotide probe corresponding to the -76/-41 region of the hOAT3 promoter, which includes the HNF1 binding motif and the nuclear extracts from HepG2, Caco-2, and HEK293 cells to show direct binding of HNF1 $\alpha$  or HNF1 $\beta$  to the promoter (Fig. 6A). Incubation of the -76/-41 oligonucleotide probe with nuclear extracts from three cell lines resulted in three shifted bands (*a*, *b*, and *c*). The band *a* was observed when the probe was incubated with the nuclear extract from HepG2 cells (lane 2), and an additional band (band *b*) showing faster mobility was detected when the probe was incubated with the nuclear extracts from Caco-2 cells (lane 5). Band *c* was produced by incubation with the nuclear extract from each cell line (lanes 2, 5, and 8). The formation of bands *a* and *b* was abolished by an excess of unlabeled HNF1 consensus oligonucleotide (per) (lanes 3 and 6), but it was unaffected by the mutated oligonucleotide (mut) (lanes 4 and 7). These results suggest that the bands *a* and *b* can be ascribed to the binding of HNF1 $\alpha$  or HNF1 $\beta$  to the -76/-41 oligonucleotide probe.

To confirm the specificity of HNF1 binding, supershift analysis was performed with HepG2 and Caco-2 nuclear extracts using a specific antibody to HNF1 $\alpha$  or HNF1 $\beta$  (Fig. 6B). Band *a* was supershifted by the addition of an antibody to HNF1 $\alpha$  but not by an antibody to HNF1 $\beta$  (lanes 3, 4, 6, and 7), whereas band *b* observed only in Caco-2 cells was supershifted by the addition of either of the antibodies (lanes 6 and 7). Thus, band *a* corresponds to HNF1 $\alpha$ /HNF1 $\alpha$  homodimer, and band *b* corresponds to HNF1 $\alpha$ /HNF1 $\beta$  heterodimer. A previous report has shown that HNF1 $\beta$ /HNF1 $\beta$  homodimer migrating faster than HNF1 $\alpha$ /HNF1 $\beta$  heterodimer may exist when both isoforms are present (Rey-Campos et al., 1991). However, in the present study, the formation of HNF1 $\beta$ /

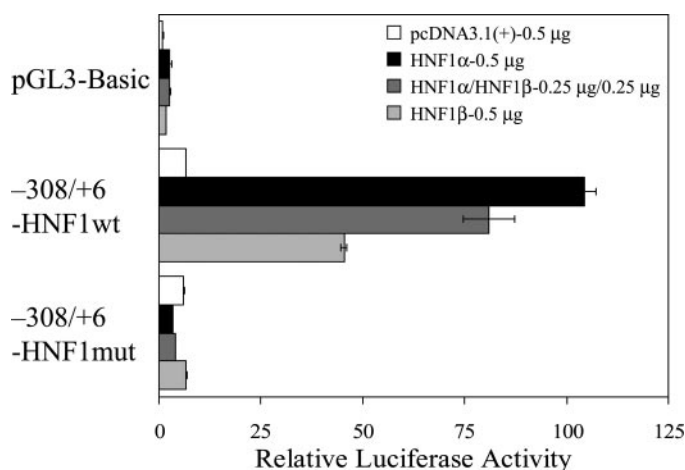


**Fig. 4.** Effect of mutations in the HNF1 binding motif on the hOAT3 promoter activity. HepG2 (white bars), Caco-2 (gray bars), and HEK293 cells (black bars) were transfected with wild-type (-308/+6-HNF1wt) or mutated (-308/+6-HNF1mut) promoter construct, and the luciferase activity was measured as described under *Materials and Methods*. The relative luciferase activity of the mutated construct is shown as a percentage of the wild-type construct in each cell line. Results are presented as the mean  $\pm$  S.E. of triplicate samples.

HNF1 $\beta$  homodimer was not clearly observed in Caco-2 cells where both isoforms were detected. The band *c* is ascribed to some nonspecific binding to the labeled probe, because this band was not abolished by the addition of unlabeled oligonucleotides. Taken together, these data suggest that the protein complex binding to the hOAT3 HNF1 motif in HepG2 cells is HNF1 $\alpha$ /HNF1 $\alpha$  homodimer and those in Caco-2 cells include HNF1 $\alpha$ /HNF1 $\beta$  heterodimer as well as HNF1 $\alpha$ /HNF1 $\alpha$  homodimer. The difference in the band pattern is probably due to the different protein expression pattern of HNF1 $\alpha$  and HNF1 $\beta$  in the two cell lines (Fig. 3B).

**Transcriptional Activation of mOat3 Promoter by HNF1 $\alpha$  and HNF1 $\beta$ .** To investigate whether HNF1 $\alpha$  or HNF1 $\beta$  is also involved in the promoter activity of mOat3, the proximal putative promoter of mOat3 was cloned into pGL3-Basic vector using the PCR-based approach, and the promoter activity of this reporter construct was measured in HepG2, Caco-2, and HEK293 cells. The luciferase activity of the mOat3 -156/+6 promoter construct (mOat3-Luc) was 6.5- and 3.6-fold higher than the promoterless pGL3-Basic plasmid in HepG2 and Caco-2 cells, respectively, whereas the activity in HEK293 cells was not significant (Supplemental Data A). Exogenous expression of HNF1 $\alpha$  or HNF1 $\beta$  in HEK293 cells enhanced the promoter activity of mOat3 (Supplemental Data B). These observations suggest that the functional importance of HNF1 $\alpha$  and HNF1 $\beta$  for the basal transcription of OAT3 genes is conserved among species.

**Repression of hOAT3 Promoter Activity by DNA Methylation.** To investigate the possible role of DNA methylation on the transcriptional repression of hOAT3, hOAT3 -308/+6 promoter construct was methylated *in vitro*; transfected into HepG2, Caco-2, and HEK293 cells; and the luciferase activity was measured. The transcriptional activity of hOAT3 was dramatically reduced by *in vitro* methylation of the promoter construct (Fig. 7). These data indicate that the



**Fig. 5.** Effect of exogenously expressed HNF1 $\alpha$  and HNF1 $\beta$  on hOAT3 promoter function in HEK293 cells. HEK293 cells were transfected with wild type (-308/+6-HNF1wt) or mutated (-308/+6-HNF1mut) promoter construct, or a promoterless pGL3-Basic plasmid, together with 0.5  $\mu$ g/well empty pcDNA3.1(+) vector (white bars), 0.5  $\mu$ g/well HNF1 $\alpha$  expression vector (black bars), 0.25  $\mu$ g/well HNF1 $\alpha$  and HNF1 $\beta$  expression vectors (gray bars), or 0.5  $\mu$ g/well HNF1 $\beta$  expression vector (light gray bars). The luciferase activity was measured as described under *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(+). Results are presented as the mean  $\pm$  S.E. of triplicate samples.



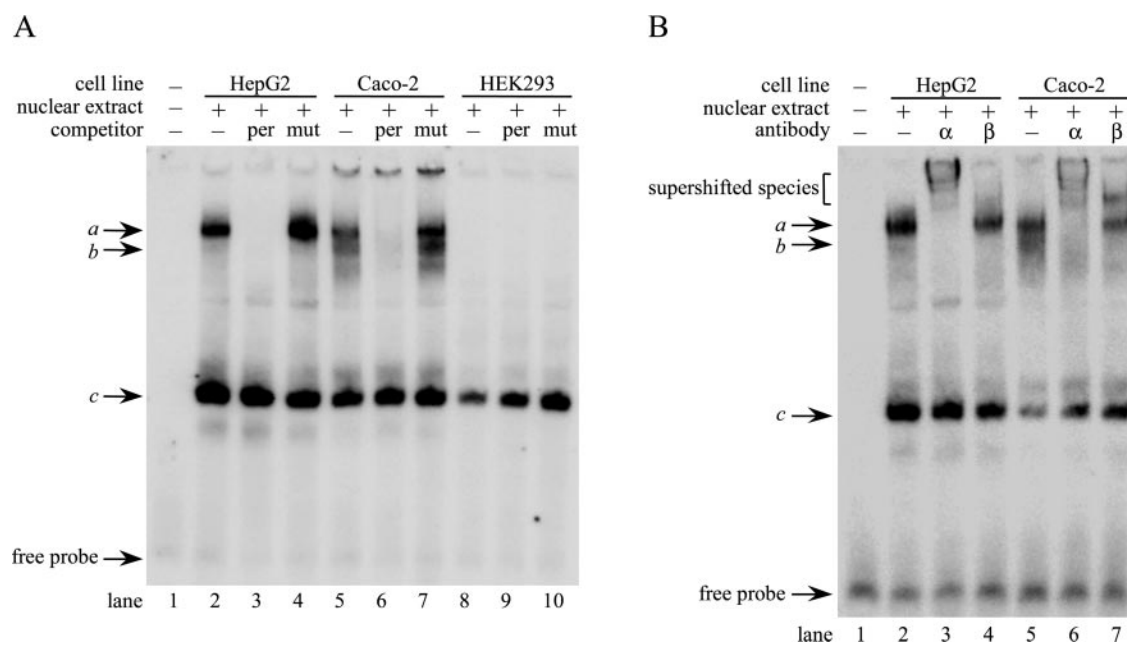
expression of hOAT3 can be suppressed at a transcriptional level by DNA methylation.

**Activation of hOAT3 Transcription by 5azadC Treatment in Nonexpressing Cell Lines.** Expression of the endogenous hOAT3 gene in HepG2, Caco-2, and HEK293 cells is below the detection limit. To investigate whether the hOAT3 gene could be activated by DNA demethylation in these nonexpressing cell lines, the total RNA from each cell line treated with 5azadC, an inhibitor for DNA methyltransferases, was subjected to RT-PCR analysis. hOAT3 mRNA became detectable in Caco-2 cells after treatment with 5azadC in a concentration-dependent manner (Fig. 8A), whereas no induction of hOAT3 mRNA was observed in HepG2 or HEK293 cells (data not shown). In HEK293 cells, however, transient transfection of HNF1 $\alpha$  alone or cotrans-

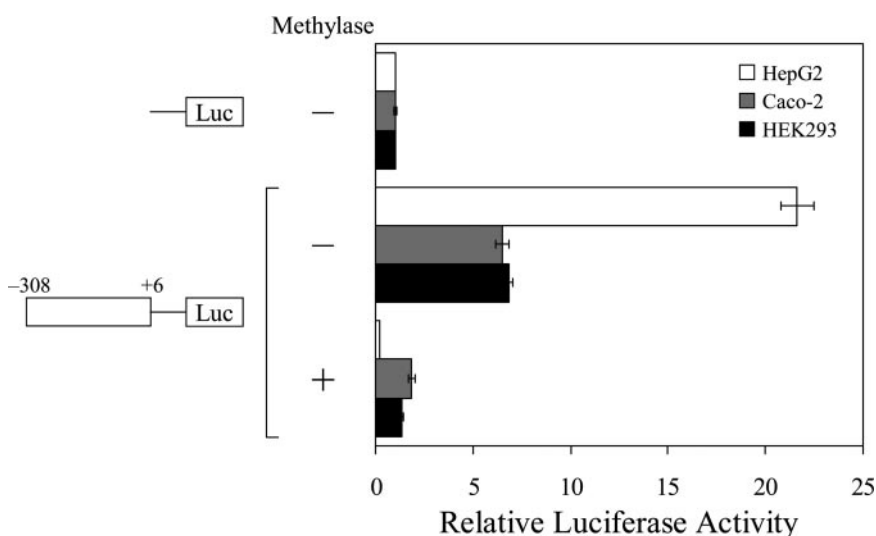
fection of HNF1 $\alpha$  and HNF1 $\beta$  followed by treatment with 100  $\mu$ M 5azadC elicited de novo expression of hOAT3 mRNA, whereas transfection of HNF1 $\beta$  alone was not sufficient to induce hOAT3 expression (Fig. 8B). These data suggest that the repression mechanism of the hOAT3 gene in hOAT3-nonexpressing cells involves DNA methylation and that synergism between HNF1 $\alpha$  or HNF1 $\beta$  expression and DNA demethylation may be required for the transcription of this gene.

## Discussion

In the present study, the transcriptional regulation of the hOAT3 gene was characterized for the first time. The promoter region required for basal promoter activity was deter-



**Fig. 6.** Electrophoretic mobility shift assay using the oligonucleotide probe corresponding to the  $-76/-41$  region and nuclear extracts from HepG2, Caco-2, and HEK293 cells. A, competition assays. Digoxigenin-labeled probe (Table 1) was incubated with nuclear extracts from HepG2, Caco-2, and HEK293 cells in the presence or absence of a 25-fold excess of unlabeled competitor (per or mut) as indicated. B, supershift analysis. The probe was incubated with nuclear extracts from HepG2 and Caco-2 cells in the presence or absence of a specific antibody against HNF1 $\alpha$  ( $\alpha$ ) or HNF1 $\beta$  ( $\beta$ ) as indicated. The DNA-protein complex was detected as described under *Materials and Methods*.



**Fig. 7.** Effect of in vitro methylation on the hOAT3 promoter activity. hOAT3  $-308/+6$  promoter construct was methylated in vitro with SssI methylase and transfected into HepG2 (white bars), Caco-2 (gray bars), and HEK293 cells (black bars). The luciferase activity was measured as described under *Materials and Methods* and was shown as the factor of induction over background activity measured in cells transfected with pGL3-Basic without methylation in each cell line. Results are presented as the mean  $\pm$  S.E. of triplicate samples.

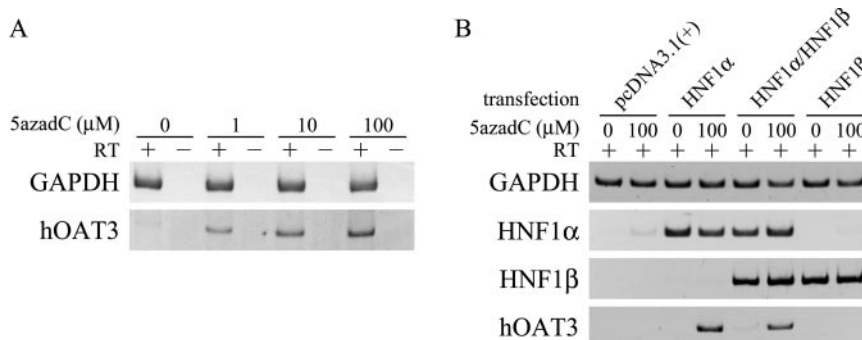
mined using a series of deleted promoter-reporter constructs (Fig. 2), and the region extending from  $-308$  to  $+6$  relative to the transcriptional start site was found to be important for promoter activity. An almost 70% homologous sequence over 300 base pairs to the hOAT3 minimal promoter was found in the mouse and rat Oat3 genomic locus. A database search with Mat Inspector revealed that the TATA motif and HNF1 motif are conserved among species as depicted in Fig. 1, suggesting the importance of these elements in the regulation of OAT3 genes.

The contribution of HNF1 $\alpha$  and HNF1 $\beta$  to the basal promoter activity of the hOAT3 gene was demonstrated by several in vitro studies. Mutations in the hOAT3 minimal promoter HNF1 motif resulted in an approximately 50% reduction in the luciferase activity compared with the wild-type construct in HepG2 and Caco-2 cells, but not in HEK293 cells (Fig. 4), which is consistent with the different expression pattern of HNF1 $\alpha$  and HNF1 $\beta$  in these cell lines (Fig. 3). The residual activity of the mutated construct may be ascribed to the transactivation by other factors, the binding of which is also affected by DNA methylation, because in vitro methylation of the minimal promoter construct almost abolished its activity (Fig. 7). The amount or contribution of these unknown factors in HEK293 cells may be higher than that in Caco-2 cells, because the luciferase activity of the hOAT3 minimal promoter in HEK293 cells is similar to that in Caco-2 cells regardless of the absence of HNF1 $\alpha$  and HNF1 $\beta$ . Further studies will be required to identify these factors that are also required for the transcription of hOAT3. Cotransfection of HNF1 $\alpha$  and/or HNF1 $\beta$  dramatically enhanced the hOAT3 wild-type promoter-driven luciferase activity in HEK293 cells, whereas the luciferase activity driven by the mutated promoter was unaffected (Fig. 5). Binding of HNF1 $\alpha$  or HNF1 $\beta$  to the HNF1 binding motif in the hOAT3 promoter was confirmed by electrophoretic mobility shift assays (Fig. 6A). Supershift analysis using specific antibodies for HNF1 $\alpha$  and HNF1 $\beta$  suggested that the protein complex binding to this region is HNF1 $\alpha$ /HNF1 $\alpha$  homodimer or HNF1 $\alpha$ /HNF1 $\beta$  heterodimer (Fig. 6B). These data indicate that HNF1 $\alpha$ , HNF1 $\beta$ , or both are critical for hOAT3 gene expression, although the transactivation potency of HNF1 $\beta$  is lower than that of HNF1 $\alpha$  (Fig. 5). It seems that the transcription of mOat3 is also under the control of HNF1 $\alpha$  and HNF1 $\beta$ . The involvement of HNF1 $\alpha$  and HNF1 $\beta$  in regulation of the OAT3

gene is conserved among species, further supporting a critical role for these transcription factors in the regulation of the hOAT3 and mOat3 genes.

hOAT3 is predominantly expressed in the kidney and only weakly in the brain and skeletal muscle (Cha et al., 2001; Alebouyeh et al., 2003). Moreover, in the kidney, the expression is restricted to the proximal tubules. Consistent with the distribution of hOAT3 in the kidney, HNF1 $\alpha$  expression is primarily detected in the proximal tubules, whereas HNF1 $\beta$  is expressed throughout all segments of the nephron, from the proximal tubules to the collecting ducts (Lazzaro et al., 1992; Pontoglio et al., 1996). However, it should be noted that the tissue distribution of HNF1 $\alpha$  and HNF1 $\beta$  is much wider than that of hOAT3 (Blumenfeld et al., 1991; Rey-Campos et al., 1991). In addition to the kidney, they are expressed in the liver, intestine, stomach, and pancreas where hOAT3 is not expressed. Therefore, the tissue-specific and region-restricted expression of hOAT3 cannot be accounted for only by HNF1 $\alpha$  and/or HNF1 $\beta$ , and other mechanisms must be involved in the regulation of hOAT3 expression.

Because multiple CpG dinucleotides are located in the hOAT3 minimal promoter region (Fig. 1), the involvement of DNA methylation in the regulation of hOAT3 expression was investigated. The transcriptional activity of the hOAT3 minimal promoter was suppressed by in vitro methylation of the reporter construct (Fig. 7). The expression of hOAT3, normally silent in Caco-2 and HEK293 cells, was activated de novo by DNA demethylation in Caco-2 cells, and by the transient transfection of HNF1 $\alpha$  or cotransfection of HNF1 $\alpha$  and HNF1 $\beta$  together with the DNA demethylation in HEK293 cells (Fig. 8). However, the expression of hOAT3 in HEK293 cells transfected with HNF1 $\beta$  alone followed by DNA demethylation with 5azadC was below the detection limit, although independent transfection of HNF1 $\beta$  enhanced the luciferase activity of hOAT3 wild-type reporter construct (Fig. 5). This could be due to the lower transactivation potency of the HNF1 $\beta$ /HNF1 $\beta$  homodimer. On the other hand, there was no induction of hOAT3 mRNA in HepG2 cells by 5azadC treatment, regardless of the presence of endogenous HNF1 $\alpha$  and HNF1 $\beta$ . The lack of inducibility of hOAT3 expression in HepG2 may be accounted for by the presence of negative regulatory factors observed predominantly in HepG2 cells in the luciferase assays (Fig. 2) and/or the absence of other transcription factors necessary for de novo



**Fig. 8.** Reactivation of the hOAT3 expression in hOAT3-negative cells. **A**, expression profile of hOAT3 mRNA in 5azadC-treated Caco-2 cells. The expression of GAPDH and hOAT3 mRNA was determined by RT-PCR in Caco-2 cells cultured for 72 h with 0, 1, 10, or 100  $\mu$ M 5azadC. **B**, expression profile of hOAT3 mRNA in HEK293 cells transfected with HNF1 $\alpha$  and/or HNF1 $\beta$  together with 5azadC treatment. The expression of GAPDH, HNF1 $\alpha$ , HNF1 $\beta$ , and hOAT3 mRNA was determined by RT-PCR in HEK293 cells transiently transfected with 0.5  $\mu$ g/well pcDNA3.1(+), 0.5  $\mu$ g/well HNF1 $\alpha$ , 0.25  $\mu$ g/well HNF1 $\alpha$  and HNF1 $\beta$ , or 0.5  $\mu$ g/well HNF1 $\beta$ , followed by treatment with 0 or 100  $\mu$ M 5azadC for 48 h. Results with the PCR templates with reverse transcription (RT +) are shown. No amplified products were detected from the templates without reverse transcription (data not shown).



expression of hOAT3. Further analysis will be required for the identification of these factors. Taken together, these observations indicate that the expression of hOAT3 is negatively regulated by DNA methylation.

There have been several reports regarding the involvement of DNA methylation in tissue-specific gene expression (Cho et al., 2001; Imamura et al., 2001; Futscher et al., 2002; Hattori et al., 2004; Jin et al., 2005). It is possible that the minimal promoter region of hOAT3 is hypermethylated in the tissues where the expression of hOAT3 is not detected, such as liver and intestine. The hypermethylation of the promoter region may render the neighboring chromatin structure inaccessible for many transcription factors, including HNF1 $\alpha$  and HNF1 $\beta$ , through deacetylation of histones. Thus, regardless of the expression of HNF1 $\alpha$  and HNF1 $\beta$ , the expression of hOAT3 is suppressed in those tissues.

The mechanism underlying the proximal tubule-restricted expression of hOAT3 in the kidney is more complicated. The nuclear extracts from the kidney normally contain HNF1 $\alpha$ /HNF1 $\beta$  heterodimers and HNF1 $\beta$ /HNF1 $\beta$  homodimers (Pontoglio et al., 1996), and the results of the supershift experiments suggest that the protein complex binding to the HNF1 motif of hOAT3 might be the HNF1 $\alpha$ /HNF1 $\alpha$  homodimer or HNF1 $\alpha$ /HNF1 $\beta$  heterodimer (Fig. 6B). It is likely that the promoter region of hOAT3 is hypomethylated in the kidney proximal tubules, enabling the HNF1 $\alpha$ /HNF1 $\beta$  heterodimers to bind to their recognition sites. This model is supported by the results showing that the expression of hOAT3 was induced by cotransfection of HNF1 $\alpha$  and HNF1 $\beta$  with the concomitant DNA demethylation in HEK293 cells (Fig. 8B). The methylation status in the other nephron segments is debatable. The promoter region may be hypermethylated in the whole nephron except for the proximal tubules, resulting in the suppression of hOAT3 expression. A second possibility is that although the promoter region is not highly methylated, interaction with the HNF1 $\beta$ /HNF1 $\beta$  homodimer is not sufficient to evoke the hOAT3 expression as observed in Fig. 8B.

Taking all these findings into consideration, it seems that the tissue specificity of the expression of hOAT3 may be explained by the coordinated action of genetic (HNF1 $\alpha$  and HNF1 $\beta$ ) and epigenetic (DNA methylation) mechanisms. Analysis of the Oat3 expression in HNF1 $\alpha$ -null mice (Pontoglio et al., 1996; Lee et al., 1998) will show the relative contribution of HNF1 $\alpha$  in vivo. In addition, direct demonstration of the difference in the DNA methylation and chromatin status in the hOAT3 promoter region among in vivo tissues/cells will be required in future studies.

The tissue distribution of mOat3 and rOat3 is not consistent with that of hOAT3: they are expressed not only in the kidney but also in the liver and the brain (Kusuhara et al., 1999; Kikuchi et al., 2003; Ohtsuki et al., 2004). It is noteworthy that the positions of the CpG dinucleotides in the hOAT3 promoter are not conserved in the corresponding region of mouse and rat Oat3 (Fig. 1). The difference in the contribution of epigenetic factors to the regulation of OAT3 gene expression may explain the species difference in the expression patterns, because the contribution of genetic factors seems to be comparable as far as HNF1 $\alpha$  and HNF1 $\beta$  are concerned.

Despite the numerous studies of the genes regulated by HNF1 $\alpha$  or HNF1 $\beta$ , there are few reports regarding the mechanism whereby these homeodomain proteins can bind to the

promoter region of their target genes or transactivate the expression in a tissue-specific manner. Here, we have provided the first evidence suggesting that the methylation profile of the promoter or the chromatin configuration of the target genes determines the accessibility of HNF1 $\alpha$  and HNF1 $\beta$ . The synergistic action of the chromatin structure in the promoter region and binding of HNF1 $\alpha$  and HNF1 $\beta$  could be a critical clue to the further understanding of the tissue-specific gene regulation by these two transcription factors. Future studies are needed to investigate whether this scheme is applicable to other genes.

In conclusion, the present study provides a clear demonstration that the expression of hOAT3 is positively regulated by HNF1 $\alpha$  and HNF1 $\beta$  and negatively regulated by DNA methylation. This is the first demonstration of the importance of HNF1 $\alpha$  and HNF1 $\beta$  in the regulation of genes indispensable for detoxification in the kidney. Furthermore, it is also suggested that the coordinated action of genetic and epigenetic factors might explain the tissue-specific expression of hOAT3.

## References

- Aleebouyeh M, Takeda M, Onozato ML, Tojo A, Noshiro R, Hasannejad H, Inatomi J, Narikawa S, Huang XL, Khamdang S, et al. (2003) Expression of human organic anion transporters in the choroid plexus and their interactions with neurotransmitter metabolites. *J Pharmacol Sci* **93**:430–436.
- Bai Y, Pontoglio M, Hiesberger T, Sinclair AM, and Igarashi P (2002) Regulation of kidney-specific Ksp-cadherin gene promoter by hepatocyte nuclear factor-1beta. *Am J Physiol* **283**:F839–F851.
- Barbacci E, Reber M, Ott MO, Breillat C, Huetz F, and Cereghini S (1999) Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. *Development* **126**:4795–4805.
- Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes Dev* **16**:21–21.
- Blumenfeld M, Maury M, Chouard T, Yaniv M, and Condamine H (1991) Hepatic nuclear factor 1 (HNF1) shows a wider distribution than products of its known target genes in developing mouse. *Development* **113**:589–599.
- Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, and Endou H (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59**:1277–1286.
- Cheret C, Doyen A, Yaniv M, and Pontoglio M (2002) Hepatocyte nuclear factor 1 alpha controls renal expression of the Npt1-Npt4 anionic transporter locus. *J Mol Biol* **322**:929–941.
- Cho JH, Kimura H, Minami T, Ohgane J, Hattori N, Tanaka S, and Shiota K (2001) DNA methylation regulates placental lactogen I gene expression. *Endocrinology* **142**:3389–3396.
- Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, Pontoglio M, Yaniv M, and Barra J (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Morphogenesis* **129**:1829–1838.
- Coffinier C, Thepot D, Babinet C, Yaniv M, and Barra J (1999) Essential role for the homeoprotein vHNF1/HNF1beta in visceral endoderm differentiation. *Development* **126**:4785–4794.
- Eraly SA, Vallon V, Vaughn DA, Gangotri JA, Richter K, Nagle M, Monte JC, Rieg T, Truong DM, Long JM, et al. (2006) Decreased renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knock-out mice. *J Biol Chem* **281**:5072–5083.
- Futscher BW, Oshiro MM, Wozniak RJ, Holtan N, Hanigan CL, Duan H, and Domann FE (2002) Role for DNA methylation in the control of cell type specific maspin expression. *Nat Genet* **31**:175–179.
- Gresh L, Fischer E, Reimann A, Tanguy M, Garbay S, Shao X, Hiesberger T, Fiette L, Igarashi P, Yaniv M, et al. (2004) A transcriptional network in polycystic kidney disease. *EMBO (Eur Mol Biol Organ) J* **23**:1657–1668.
- Hasegawa M, Kusuhara H, Endou H, and Sugiyama Y (2003) Contribution of organic anion transporters to the renal uptake of anionic compounds and nucleoside derivatives in rat. *J Pharmacol Exp Ther* **305**:1087–1097.
- Hasegawa M, Kusuhara H, Sugiyama D, Ito K, Ueda S, Endou H, and Sugiyama Y (2002) Functional involvement of rat organic anion transporter 3 (rOat3; Slc22a8) in the renal uptake of organic anions. *J Pharmacol Exp Ther* **300**:746–753.
- Hattori N, Nishino K, Ko YG, Ohgane J, Tanaka S, and Shiota K (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J Biol Chem* **279**:17063–17069.
- Imamura T, Ohgane J, Ito S, Ogawa T, Hattori N, Tanaka S, and Shiota K (2001) CpG island of rat sphingosine kinase-1 gene: tissue-dependent DNA methylation status and multiple alternative first exons. *Genomics* **76**:117–125.
- Jin B, Seong JK, and Ryu DY (2005) Tissue-specific and de novo promoter methylation of the mouse glucose transporter 2. *Biol Pharm Bull* **28**:2054–2057.
- Jung D, Hagenbuch B, Gresh L, Pontoglio M, Meier PJ, and Kullak-Ublick GA (2001) Characterization of the human OATP-C (SLC21A6) gene promoter and regulation of liver-specific OATP genes by hepatocyte nuclear factor 1  $\alpha$ . *J Biol Chem* **276**:37206–37214.

- Kikuchi R, Kusuhara H, Sugiyama D, and Sugiyama Y (2003) Contribution of organic anion transporter 3 (Slc22a8) to the elimination of *p*-aminohippuric acid and benzylpenicillin across the blood-brain barrier. *J Pharmacol Exp Ther* **306**: 51–58.
- Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH, Sugiyama Y, Kanai Y, and Endou H (1999) Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. *J Biol Chem* **274**:13675–13680.
- Lazzaro D, De Simone V, De Magistris L, Lehtonen E, and Cortese R (1992) LFB1 and LFB3 homeoproteins are sequentially expressed during kidney development. *Development* **114**:469–479.
- Lee YH, Sauer B, and Gonzalez FJ (1998) Laron dwarfism and non-insulin-dependent diabetes mellitus in the Hnf-1alpha knockout mouse. *Mol Cell Biol* **18**:3059–3068.
- Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**:265–275.
- Mendel DB and Crabtree GR (1991) HNF-1, a member of a novel class of dimerizing homeodomain proteins. *J Biol Chem* **266**:677–680.
- Mendel DB, Hansen LP, Graves MK, Conley PB, and Crabtree GR (1991) HNF-1 alpha and HNF-1 beta (vHNF-1) share dimerization and homeo domains, but not activation domains, and form heterodimers in vitro. *Genes Dev* **5**:1042–1056.
- Ohtsuki S, Kikkawa T, Mori S, Hori S, Takanaga H, Otagiri M, and Terasaki T (2004) 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.
- Pontoglio M, Barra J, Hadchouel M, Doyen A, Kress C, Bach JP, Babinet C, and Yaniv M (1996) Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell* **84**:575–585.
- Pontoglio M, Prie D, Cheret C, Doyen A, Leroy C, Froguel P, Velho G, Yaniv M, and Friedlander G (2000) HNF1alpha controls renal glucose reabsorption in mouse and man. *EMBO (Eur Mol Biol Organ) Rep* **1**:359–365.
- Rey-Campos J, Chouard T, Yaniv M, and Cereghini S (1991) vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1. *EMBO (Eur Mol Biol Organ) J* **10**:1445–1457.
- Robertson EE and Rankin GO (2005) Human renal organic anion transporters: characteristics and contributions to drug and drug metabolite excretion. *Pharmacol Ther* **109**:399–412.
- Sekine T, Miyazaki H, and Endou H. 2006. Molecular physiology of renal organic anion transporters. *Am J Physiol* **290**:F251–F261.
- Shih DQ, Bussen M, Sehayek E, Ananthanarayanan M, Shneider BL, Suchy FJ, Shefer S, Bollileni JS, Gonzalez FJ, Breslow JL, et al. (2001) Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism. *Nat Genet* **27**:375–382.
- Shiota K (2004) DNA methylation profiles of CpG islands for cellular differentiation and development in mammals. *Cytogenet Genome Res* **105**:325–334.
- Sweet DH, Miller DS, Pritchard JB, Fujiwara Y, Beier DR, and Nigam SK (2002) Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (*Oat3* (*Slc22a8*)) knockout mice. *J Biol Chem* **277**:26934–26943.
- Tronche F and Yaniv M (1992) HNF1, a homeoprotein member of the hepatic transcription regulatory network. *Bioessays* **14**:579–587.
- Van Aubel RA, Masereeuw R, and Russel FG (2000) Molecular pharmacology of renal organic anion transporters. *Am J Physiol* **279**:F216–F232.

**Address correspondence to:** Dr. Yuichi Sugiyama, Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: sugiyama@mol.f.u-tokyo.ac.jp