Characterization of the Human OATP-C (SLC21A6) Gene Promoter and Regulation of Liver-specific OATP Genes by Hepatocyte Nuclear Factor 1α*

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OATP-C (SLC21A6) is the predominant Na\(^+\)-independent uptake system for bile salts and bilirubin of human liver and is expressed exclusively at the basolateral (sinusoidal) hepatocyte membrane. To investigate the basis of liver-specific expression of OATP-C, we studied promoter function in the two hepatocyte-derived cell lines HepG2 and Huh7 and in nonhepatic HeLa cells. OATP-C promoter constructs containing from 66 to 950 nucleotides of 5' regulatory sequence were active in HepG2 and Huh7 but not HeLa cells, indicating that determinants of hepatocyte-specific expression reside within the minimal promoter. Deoxyribonuclease I footprint analysis revealed a single region that was protected by HepG2 and Huh7 but not HeLa cell nuclear extracts. The liver-enriched transcription factor hepatocyte nuclear factor 1α (HNF1α) was shown by mobility shift assays to bind within this footprint. Coexpression of HNF1α stimulated OATP-C promoter activity 30-fold in HepG2 and 49-fold in HeLa cells. Mutation of the HNF1 site abolished promoter function, indicating that HNF1α is critical for hepatocyte-specific OATP-C gene expression. The human OATP8 (SLC21A8) and mouse Oatp4 (Slc21a6) promoters were also responsive to HNF1α coexpression in HepG2 cells. These data support a role for HNF1α as a global regulator of liver-specific bile salt and organic anion transporter genes.

A key function of the liver is the extraction and excretion of numerous endogenous and xenobiotic compounds from sinusoidal blood plasma. Hepatic uptake of bile salts, conjugated steroids, selected peptides, and several anionic and cationic substrates is mediated by a family of organic anion transporting polypeptides (OATPs)\(^1\) localized at the basolateral membrane of hepatocytes. In human liver, four representatives of the OATP family have been identified (1). OATP-C (SLC21a6)\(^1\,\,2\) belongs to a liver-specific subfamily of OATPs that is expressed exclusively in hepatocytes (2–5) and was originally termed “liver-specific transporter 1” (LST-1) (3) or OATP2 (2, 4). OATP-C consists of 691 amino acids and exhibits a broad substrate specificity for numerous organic anions including the bile salts taurocholate, glycocholate, and cholate (1, 3, 4, 6), unconjugated and glucuronidated bilirubin (2, 6), dehydroepiandrosterone-3-sulfate (3, 4), estradiol-17β-glucuronide (2), estrone-3-sulfate (1), leukotriene C\(_4\) and prostaglandin E\(_2\) (1, 3), pravastatin (4), bromosulphophthalein (1, 6), the thyroid hormones triiodothyronine and thyroxine (1, 3), and the anionic cyclic peptides [\(\alpha\)-penicillamine\(^{2,5}\)]-enkephalin and BQ-123 (1). Many of these substrates are shared by other OATPs expressed in human liver (OATP-A (SLC21A9), OATP-B (SLC21A9), and OATP8 (SLC21A8)) (1); however, a substrate specific for OATP-C is unconjugated bilirubin (6).

Although no disease state that is associated with mutations in the OATP-C gene has been identified so far, OATP-C probably represents the liver-specific bile salt and bilirubin uptake system of human liver. Reduced expression of OATP-C has been observed in the chronic cholestatic liver disease primary sclerosing cholangitis (7). The rat orthologue of OATP-C, called Oatp4 (Slc21a6), exhibits reduced mRNA levels after bile duct ligation or cecum ligation and puncture, two experimental models of cholestasis (8). Cecum ligation and puncture leads to bacteremia and consequent endotoxinemia, which causes intrahepatic cholestasis through decreased expression of hepato cellular bile salt and organic anion transporters (9). In rats, endotoxinemia decreases nuclear activity of hepatocyte nuclear factor 1 (HNF1)\(^1\), a critical factor for basal expression of the Na\(^+\)-taurocholate cotransporting polypeptide (Ntcp\(^1\) (Slc10a1)) (10). Whether reduced nuclear binding of HNF1 is also the cause of decreased hepatic Oatp4 expression in endotoxemic rats is unknown.

Using oligonucleotide microchip expression analysis, mRNA levels of Ntcp (Slc10a1), Oatp1 (Slc21a1), Oatp2 (Slc21a5), and Oatp4 (Slc21a6) were found to be decreased or absent in the livers of Tcf1\(^−\)− (HNF1α\(^−\)−) mice with a null mutation in the HNF1α gene (11). These data together with the known dependence of rat Ntcp (Slc10a1) gene expression upon HNF1α (12), suggested a general role for HNF1α as a transcriptional acti...
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Valor of hepatic bile salt and bilirubin transporters. Although a direct role for HNF1α in regulating human liver OATP genes has not been shown, two lines of evidence suggested OATP-C as a likely candidate for regulation by HNF1α as follows. (i) OATP-C is expressed only in hepatocytes and binding sites for HNF1α have been shown in the promoters or enhancers of numerous genes expressed exclusively in the liver (13, 14); (ii) the phenotype of increased serum bile acid concentrations and a 3–10-fold elevation of serum bilirubin in HNF1α-deficient (Tcf1−/−) mice (11, 15) is consistent with a reduced function of Oatp4 (Slc21a6), the liver-specific mouse orthologue of human OATP-C (SLC21A6). In this study, we report the isolation of the human OATP-C promoter and demonstrate the critical role of HNF1α for liver-specific OATP gene expression.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]Adenosine triphosphate (3,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Restriction enzymes and proteinase K were from Roche Molecular Biochemicals, PfuTurbo DNA polymerase were from Life Technologies, Inc., and T4 polynucleotide kinase was from Stratagene (Amsterdam, Netherlands). Polyacrylamide was obtained from Bio-Rad. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich.

Localization of the 5′-Region of the OATP-C Gene—Total RNA was isolated from human liver by the acid guanidinium/phenol/chloroform procedure (16). The 5′-end of the OATP-C mRNA was determined using rapid amplification of cDNA ends (5′-RACE’ system, Roche Molecular Biochemicals). 1 μg of total RNA was reverse-transcribed using the OATP-C-specific primer C-RT (see Table I). The 5′-end was subsequently amplified by PCR using primer C-PCRace and nested primer nC-PCRace as downstream primers (see Table I). The resulting 5′-RACE product was subcloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) that had been predigested with Bgl II and Sac I and subsequently digested with Sac I of LipofectAMINE 2000 (Life Technologies, Inc.) and 0.5 μg of nuclear extract (Qiagen, Basel, Switzerland). 1 ng of labeled PCR fragment was incubated with 10 μl of nuclear extracts and 5 μl of a 50 μl of buffer (0.5 M saccharose, 15 mM Tris/HCl, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 5 mM MgCl2, 25 mM CaCl2). After the addition of 1 volume of digestion buffer (5 mM MgCl2, 2.5 mM CaCl2), a 60-min digestion with 5 μl of freshly diluted DNase I (Roche Molecular Biochemicals) was performed. Digestion was terminated by the addition of 1 volume of stop solution (50 mM EDTA, 0.1% SDS, 150 μg/ml RNA, 200 μg/ml proteinase K). DNase I-digested samples and a control sample digested in the absence of nuclear proteins were extracted with phenol/chloroform and precipitated in ethanol. Resuspended DNA samples were loaded on a denaturing polyacrylamide gel alongside a sequencing reaction that had employed the Cy3-p+30 primer and the C-424 plasmid as template. Bands were detected and analyzed using the AlfExpress system (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotide probes were obtained by hybridizing single-stranded complementary oligonucleotides (Microsynth, Balgach, Switzerland). Dimers with the sense sequences shown in Table I were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Stratagene, Amsterdam, Netherlands). Sequence wtHNF1 corresponded to the wild type HNF1α sequence found in the OATP-C gene promoter, perHNF1 corresponded to a perfect consensus sequence for HNF1, and mutHNF1 corresponded to the wild type sequence mutated within the HNF1 recognition site. For gel mobility shift assays, 5 μg of nuclear extracts were incubated on ice for 20 min with 2–5 fmol of [γ-32P]-end-labeled dimerized oligonucleotide and 1 μg of poly(dI)poly(dC) (Amersham Pharmacia Biotech) in 20 mM HEPES-KOH, pH 7.9, 20% glycerol, 100 mM KCl, 2 mM MgCl2, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. For competition assays, a 10–500-fold excess of unlabeled dimerized oligonucleotides was added. For supershift experiments, 1 μl of antibody against HNF1α (17), 1 μl of antibody against HNF1β (18), or 2 μl of antibody against HNF1α (H-206; sc-9886, Santa Cruz Biotechnology Inc., Heidelberg, Germany) was added to the reaction mix. Reactions were analyzed by electrophoresis through 3.4% polyacrylamide gels in 0.25× Tris-borate EDTA buffer at 120 V for 2 h.

Statistical Analysis—Reporter gene activities are expressed as the mean ± S.D. of at least three independent transfection experiments. All data were reproduced at least once using two different preparations of plasmid DNA.

RESULTS

Localization of the Transcription Initiation Site and Promoter Region of the OATP-C (SLC21A6) Gene—To identify the promoter region of the OATP-C gene, the transcription initiation site was localized by a 5′-RACE approach. Using a downstream oligonucleotide that corresponded to nucleotides 163–186 of the OATP-C cDNA sequence as published by Hisagi et al. (accession number AF205071) (4), and to nt 92–115 of exon 1 of the OATP-C gene, as published by König et al. (AJ400749) (5), a single PCR product was amplified. Sequence analysis indicated that eight additional nucleotides (AAAGGGTG) are present in the 5′-untranslated region compared with the cDNA sequence reported by Hisagi et al. (4). These additional nucleotides were also present in the genomic SLC21A6 sequence derived from BAC clone RP11–12505G (AC022383) and were consequently designated as the transcription start site: +1 base. As Blast and BLASTX analysis of the genomic SLC21A6 gene identifies an additional exon with a length of 39 bp (exon –1 in Fig. 1A) is a member of a 10277-bp intronic sequence from the 66-bp untranslated region of exon 1 (AJ400749) (5). The transcription start site of the OATP-C cDNA is located 105 bp upstream of the ATG initiation codon.

Analysis of the 5′-Flanking Region of the Human OATP-C
The 5′-flanking region of the OATP-C (SLC21A6) gene was PCR-amplified from human genomic DNA and showed sequence identity with clone Homo sapiens 12p BAC RP11–125O5 (AC022335). Fig. 1B displays the sequence from nt −950 to +40 relative to the transcription initiation site. Homologies to known gene regulatory elements were identified using the program MatInspector (Genomatix Software, Munich, Germany). Several potential transcription factor recognition sites were found including ubiquitously expressed factors such as the activator protein 1 (AP-1) at nt −58 to −68 and −557 to −567. A TATA motif was identified at nt −62 to −96 but not within the immediate 5′-flanking region of the transcriptional start site. Other more generally represented DNA elements included potential binding sites for the nuclear factor 1 (NF-1) at nt −15 to −32 and −111 to −125, a CAAT motif at −199 to −206, and an octamer binding site (Oct-1) at −322 to −336.

In addition, several liver-enriched transcription factor binding sites were detected, including two sites for HNF1 at nt −39 to −51 and −165 to −177, two CAAT-enhancer binding protein (CEBP-β) binding sites at −188 to −202 and −473 to −486, and three HNF3β binding sites at −206 to −220, −639 to −653, and −829 to −842. Regarding the HNF1 binding sites, the element nearer the transcriptional start site (nt −39 to −51) showed a better match with the consensus sequence for HNF1 binding than the element located further upstream (nt −165 to −177).

Analysis of Basal OATP-C (SLC21A6) Gene Promoter Activity in Cell Lines of Hepatocellular and Non-hepatocellular Origin—Human OATP-C gene expression is limited strictly to hepatocytes (2). To identify the cis-acting elements in the OATP-C promoter that control liver-specific transcriptional regulation, the hepatocyte-derived cell lines HepG2 and Huh7 and the nonhepatic HeLa cell line were transfected with plasmids C-950, C-424, C-128, and C-66 that contained deletion fragments of the OATP-C promoter sequence (see Fig. 1B). In HepG2 and Huh7 cells, all four constructs conferred significant reporter gene activity as compared with the promoterless pGL3-Basic plasmid (Fig. 2). In contrast to the hepatocyte-derived cell lines HepG2 and Huh7, no luciferase activity was induced by any of the promoter constructs in HeLa cells. These data supported the notion that a liver-enriched factor present only in HepG2 and Huh7 but not in HeLa cells is required for function of the OATP-C promoter. Whereas the fragment from −66 to +21 was sufficient to confer residual promoter activity.
Regulation of Liver-specific OATPs by HNF1α

**HNF1 Binds to the OATP-C Promoter**—The identity of the factor that was found to bind in the −37/−59 region by footprint analysis was investigated by electrophoretic mobility shift assays. A 32P-labeled dimerized oligonucleotide corresponding to nt −32 to −58 (wtHNF1) was incubated with nuclear proteins from HepG2, Huh7, and HeLa cells. Binding of nuclear proteins was only found with nuclear extracts from HepG2 and Huh7 cells, resulting in a shifted band (Fig. 4). No binding occurred with HeLa cell nuclear extracts. To study whether the bound protein exhibited the same electrophoretic migration pattern as HNF1α, a 32P-labeled dimerized oligonucleotide with a perfect palindromic HNF1 binding site (perHNF1) was incubated with HepG2, Huh7, and HeLa cell nuclear extracts. The binding pattern of the perfect HNF1 consensus oligonucleotide was identical to that of the OATP-C-derived oligonucleotide, strongly suggesting that the protein binding to the OATP-C sequence was HNF1. The DNA-protein complex was not formed in the presence of an excess of unlabeled perHNF1 oligonucleotide but was unaffected by the presence of excess mutHNF1 oligonucleotide (Fig. 4) with a mutated HNF1 binding site (Table I). These competition experiments were repeated using increasing amounts of unlabeled wild type (wtHNF1) or mutated (mutHNF1) oligonucleotide. As shown in Fig. 5A, reduced binding of HepG2 nuclear extracts to labeled wtHNF1 oligonucleotide was already seen in the presence of a 25-fold excess of unlabeled wtHNF1 oligonucleotide and was maximal with a 200-fold excess, whereas a 100-fold or 200-fold excess of mutated mutHNF1 oligonucleotide had no effect on protein binding.

To confirm the specificity of HNF1 binding, supershift analysis was performed. The addition of antibody specifically recognizing HNF1α (17) produced a single supershifted complex (upper arrow in Fig. 5B). For control purposes, a commercial competitive antibody recognizing HNF1 was also employed and completely inhibited DNA-protein complex formation (HNF1α) in Fig. 5B). A residual lower band was not shifted by the HNF1α antibody and remained visible in the presence of the competitive HNF1 antibody (lower arrow in Fig. 5B). To exclude the possibility that this residual band represented binding of HNF1β, an antibody targeted against HNF1β (19) was employed. The HNF1β antibody also failed to shift the lower band, which thus corresponded to an unknown factor. The slight shift of the HNF1α-DNA complex induced by HNF1β antibody was attributable to the known cross-reactivity of the HNF1β antibody with HNF1α. Taken together, the data indicate that the protein complex binding to the OATP-C wtHNF1 sequence is mostly HNF1α.

In addition to supershift analysis, specificity of HNF1 binding was studied in a transfection assay. HepG2 cells were transfected with the C-128 construct and the HNF1α expression plasmid together with 1 or 10 pmol of either the wtHNF1 (−32/−58) or the mutHNF1 dimerized oligonucleotides used previously in mobility shift assays. In the presence of 10 pmol of wtHNF1 oligonucleotide, the activity of the C-128 construct was reduced to 32% of the value measured in the presence of 1 pmol wtHNF1 (data not shown). In contrast, cotransfection of
corresponded to the consensus recognition site of nuclear factor 1 (NF-1). A second region spanning nt −111 to −125 was protected by nuclear extracts from HepG2 or Huh7 cells, the sequence of which is shown (HNF1). A second region spanning nt −111 to −125 was protected by nuclear extracts from all three cell lines and corresponded to the consensus recognition site of nuclear factor 1 (NF-1).

FIG. 3. Non-radioactive DNase I footprint analysis of the immediate 5′-flanking region of the human OATP-C gene. A Cy3 single end-labeled 454-bp fragment spanning nt −424 to +30 relative to the transcription start site was incubated in the presence (HepG2, Huh7, HeLa) or absence (no extract) of nuclear extracts from the indicated cell lines and digested with DNase I. Samples were loaded alongside a sequencing reaction primed by the Cy3-p +30 oligonucleotide (A, C, G, T). Compared with the pattern obtained with nonhepatic HeLa cell nuclear extracts, a single protected region spanning nt −37 to −59 was found using nuclear extracts from HepG2 or Huh7 cells, the sequence of which is shown (HNF1). A second region spanning nt −111 to −125 was protected by nuclear extracts from all three cell lines and corresponded to the consensus recognition site of nuclear factor 1 (NF-1).

the mutHNF1 oligonucleotide had no effect on promoter activity, which was in agreement with the absence of DNA-protein complex formation by mutHNF1 (Figs. 4 and 5A). These data indicate that the −32/−58 sequence of the OATP-C promoter binds HNF1α not only in mobility shift assays but also in the intact environment of living HepG2 cells.

HNF1α Is a Potent Transactivator of the OATP-C Promoter—To investigate whether exogenously expressed HNF1α affects OATP-C promoter function in hepatocyte-derived and nonhepatic cells, an expression plasmid coding for HNF1α was introduced into HepG2 and HeLa cells together with several OATP-C promoter constructs. As shown in Fig. 6A, cotransfection of the HNF1α plasmid enhanced OATP-C promoter-driven luciferase activity 2.7-fold (C-66 construct) to 30-fold (C-424 construct) in HepG2 cells. A similar effect was observed in Huh7 cells (data not shown). Interestingly, cotransfection of HeLa cells with HNF1α plasmid produced significant activity of the OATP-C promoter (Fig. 6B). The factor of induction over luciferase activity measured in the absence of HNF1α ranged from 9.7-fold (C-66 construct) to 49-fold (C-128 construct). No additional enhancement compared with the C-128 construct was seen with the C-424 construct in HeLa cells. These data indicate that expression of HNF1α is sufficient to confer basal promoter activity of the liver-specific OATP-C gene even in the nonhepatic HeLa cell line.

The fact that the C-128 construct was induced more strongly than the C-66 construct may have been attributable to the requirement of an additional essential factor binding to the region between nt −66 and −128. This could represent nuclear factor 1, shown by footprint analysis to bind to the mutHNF1 oligonucleotide site (Table I). Introduction of these mutations had been shown to abolish HNF1 binding in mobility shift assays (Figs. 4 and 5A).

FIG. 4. Electrophoretic mobility shift assay using oligonucleotides of the −32/−58 region and nuclear extracts from HepG2, Huh7, and HeLa cells. The wtHNF1 oligonucleotide (Table I) corresponded to the wild type OATP-C-derived sequence. A DNA-protein complex that resulted in a band shift (arrow) was formed in the presence of HepG2 or Huh7 but not HeLa cell nuclear proteins. The same complex was found using a modified oligonucleotide that contained the perfect HNF1 consensus binding site (perHNF1). Competition experiments using a 500-fold excess of unlabeled perHNF1 oligonucleotide showed complete inhibition of wtHNF1 binding to nuclear proteins, whereas a 500-fold excess of an oligonucleotide that contained a mutated HNF1 recognition site (mutHNF1, see Table I) had no effect.

| Cell line | HepG2 | HeLa | Huh7 |
|-----------|-------|------|------|
| Probe | wtHNF1 | + | + | + |
| | perHNF1 | + | + | + |
| Competitor | mutHNF1 | + | + | + |
| | perHNF1 | + | + | + |

Mutagenesis of the HNF1 Binding Site Results in a Complete Loss of Activation—To assess the importance of HNF1 binding for basal promoter function, mutations were introduced into the HNF1 binding site of the −128/+21 construct by site-directed mutagenesis. The resulting reporter gene plasmid mutC-128 contained the same mutations in the −40/−49 region as the mutHNF1 oligonucleotide (Table I). Introduction of these mutations had been shown to abolish HNF1 binding in mobility shift assays (Figs. 4 and 5A).

As illustrated in Fig. 7, mutation of the HNF1 binding site resulted in a complete loss of basal promoter activity of the −128/+21 construct in HepG2 cells. Furthermore, OATP-C promoter activity could no longer be stimulated by cotransfection of HNF1α expression plasmid. In HeLa cells, activation of basal activity of the −128/+21 construct by coexpressed HNF1α was similarly absent when the mutC-128 plasmid was used (data not shown). These data provide definite evidence for the critical role of HNF1α binding to the OATP-C gene for basal promoter function.
Regulation of Liver-specific OATPs by HNF1α

**TABLE I**

Oligonucleotides used for 5′-RACE, chimeric plasmid construction, deoxyribonuclease footprinting, and mobility shift assays

| Oligonucleotide          | Sequence (5′ to 3′)                  |
|--------------------------|-------------------------------------|
| OATP-C                   |                                      |
| C-RT                     | CCCTATTCCACGAAGCATATTACCC            |
| C-PCRace                 | AGCAGTCAAAACACCTCCCAATCC            |
| nC-PCRace                | CTCCTGAAAGTTGCTCCCTGCGT            |
| p-950                    | ACCCAAGATAGAGCTCATACTAGATAAC        |
| p-424                    | TATTTCCAGGAGTCTCCAGTCTCC           |
| p-128                    | TAGAAAATGTAGCTCAAAATGTTGCC          |
| p-66                     | GACTAGAGCTTGTACATATAAGG             |
| p+21                     | GAATCTTAGATCTACTGCAAGATCCACC        |
| Cy3-p+30                 | CCTGACGCAAAGTCCACC                 |
| OATP8                    | GATAGGCTCTGGGTTAGCTCCTAG           |
| p8−120                   | GAATGCTACAGATCTCAAGATTCCTTCATC     |
| p8+38                    | CCTACCTAGGCTCATTGTGCTCTTTTC        |
| mOatp4                   | CTCAGGCTCTACAGATCTGCGCTGATCTC      |
| p4−137                   | GAGCTAGAGCTCTGTTTGCTTCT            |
| p4+15                    | HNF1α consensus sequence            |
| wtHNF1                   | GTTAATTNATTAAC                     |
| perHNF1                  | TAAAGTGTGTATTACACTGCGACTTG         |
| mutHNF1                  | TAAGGTTGTTATACACTGCGACTTG          |

**Fig. 5.** Mobility shift competition assay and supershift analysis of the wtHNF1 bound protein complex. A, competition of HepG2 nuclear protein binding to the 32P-labeled wtHNF1 oligonucleotide was already evident in the presence of a 25-fold excess of unlabeled wtHNF1 oligonucleotide and was maximal using a 200-fold excess (arrow). In contrast, a 100- or 200-fold excess of mutated mutHNF1 oligonucleotide had no effect. B, supershift analysis using a specific antibody against HNF1α (HNF1α), a commercial competitive antibody against HNF1β (HNF1β-cr. α) or an antibody targeted against HNF1β with known cross-reactivity against HNF1α (HNF1β-cr. α). The HNF1α antibody shifted most of the wtHNF1 bound protein complex (top arrow), indicating that HNF1α is the major protein binding to this region.

**HNF1α Transactivates the Human OATP8 (SLC21A8) and Mouse Oatp4 (Slc21a6) Promoters**—A homologue of OATP-C that exhibits 80% identity at the amino acid level and is also expressed exclusively in hepatocytes is human OATP8 (SLC21A8). In rodents, the only known orthologue of these two closely related human OATPs is Oatp4 (Slc21a6). Both human OATP8 and mouse Oatp4 possess a consensus recognition site for HNF1 in their promoter regions (Table II). To study whether HNF1α also affects promoter function of hOATP8 and mOatp4, HepG2 cells were transfected with promoter constructs together with the HNF1α expression plasmid. As shown in Fig. 8, coexpression of HNF1α increased both hOATP8 and mOatp4 promoter activity 3.5-fold. These data indicate positive regulation not only of OATP-C but also of other liver-specific OATP genes by HNF1α.

**DISCUSSION**

The present study reports the characterization of the promoter region of the human OATP-C (SLC21A6) gene and the identification of a binding site for the liver-enriched transcription factor HNF1α. Initially the transcription initiation site was localized by 5′-RACE and was shown to reside eight nu-
cleotides upstream of the published start of the OATP-C cDNA sequence (Fig. 1B) (4). The 5′-untranslated region extends over two exons that are separated by a 10,277-bp intron (Fig. 1A). The promoter region contains several consensus recognition sites for both ubiquitously expressed and liver-enriched transcription factors including HNF1, HNF3, CCAAT-enhancer binding protein, and activator protein 1. Basal promoter activity was observed in two hepatocyte-derived cell lines, HepG2 and Huh7, whereas the nonhepatic HeLa cell line did not express reporter gene activity from an OATP-C promoter construct (Fig. 2). To identify which nuclear factor could be responsible for liver-restricted expression of OATP-C, DNase I footprinting was performed. A clear discrimination between HeLa cells and the hepatocyte-derived cell lines was only possible with respect to a single region from nt −37 to −59 that was not protected by HeLa cell nuclear extracts (Fig. 3). This region contained the consensus sequence for HNF1. Binding of HNF1 to this region of the OATP-C promoter was confirmed in mobility shift assays (Figs. 4 and 5A) and by supershift analysis using an HNF1α antibody (Fig. 5B). No nuclear protein binding was observed using HeLa cell nuclear extracts (Fig. 4). Coexpression of exogenous HNF1α stimulated OATP-C promoter activity up to 30-fold in HepG2 cells and even produced basal promoter activity in HeLa cells (Fig. 6). Targeted mutation of the HNF1 binding site abolished not only inducibility of the OATP-C promoter by HNF1α but even basal promoter function in HepG2 cells (Fig. 7). These data indicate that HNF1α is critical for OATP-C gene expression in human liver.

Human OATP-C is the second basolateral transport system next to rat Ntcp (12) for which direct binding of HNF1α has been demonstrated. Both genes appear to be critically dependent upon HNF1α for basal promoter func-

![Image](https://www.jbc.org/content/7/37108/37212/F6)

**Fig. 6.** Effect of exogenous HNF1α overexpression on OATP-C promoter function in HepG2 and HeLa cells. Cells were transfected with the indicated OATP-C promoter constructs together with either an HNF1α expression plasmid (HNF1α, black bars) or with the empty pBluescript vector as a control (pB-SK, gray bars). A, coexpression of HNF1α led to a 2.7–30-fold increase in OATP-C promoter activity in HepG2 cells. B, HeLa cells showing no basal activity of the OATP-C promoter constructs (Fig. 2) produced significant OATP-C promoter function upon coexpression of HNF1α. The factor of induction compared with cells not expressing HNF1α ranged from 9.7-fold (C-66 construct) to 49-fold (C-128 construct). Cotransfection of the pBluescript vector had no effect on reporter activity. Luciferase activity is shown as the factor of induction over background measured in cells transfected with pGL3-Basic alone (Basic). Results are given as the mean ± S.D. of three transfections.

![Image](https://www.jbc.org/content/7/37108/37212/F7)

**Fig. 7.** Loss of OATP-C promoter activity by mutagenesis of the HNF1 binding site. HepG2 cells were transfected with wild type (C-128) or mutated (mutC-128) plasmid containing the −128/+21 region of the OATP-C gene and cotransfected with either HNF1α expression plasmid (HNF1α, black bars) or empty pBluescript vector as a control (pB-SK, gray bars). Mutation of the HNF1 binding site resulted in a complete loss of promoter activity and a loss of inducibility by exogenously expressed HNF1α. Luciferase activity is shown as the ratio firefly/Renilla luciferase (see “Experimental Procedures”), and data represent the mean of ± 1 S.D. of three transfection experiments.

**Table II.** Sequence alignment of HNF1 binding sites in the human OATP-C and OATP8 and mouse Oatp4 promoters

| Species   | Promoter | Gene symbol | HNF1 binding site |
|-----------|----------|-------------|-------------------|
| Human     | OATP-C   | SLC21A6     | GTTAATNATTAAAC   |
|           | OATP8    | SLC21A8     | TAATAAGGTCGTCATCTCCTCCTTTTCAAAAAGCCAA |
| Mouse     | Oatp4    | Slc21a6     | TAATAAGGTCGTCATCTCCTCCTTTTCAAAAAGCCAA |

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|         | OATP8    | SLC21A8     | TAATAAGGTCGTCATCTCCTCCTTTTCAAAAAGCCAA |
| Mouse   | Oatp4    | Slc21a6     | TAATAAGGTCGTCATCTCCTCCTTTTCAAAAAGCCAA |
HNF1 in the subfamily, possess consensus binding sites for SLC21A6 genes in HepG2 cells produces significant transactivation of HNF1/H9251 genes, both of which belong to the mouse Slc21a6/H9251 lines of evidence point toward a role for HNF1/H9251 Slc21a6 inactivation. The genomic organization of the OATP-C (SLC21A6) gene is similar to that of human OATP-A (SLC21A3) and OATP8 (SLC21A8) (5) and of mouse Oatp2 (Slc21a5) and Oatp4 (Slc21a6) (28, 29). The human OATP-A, OATP-C, and OATP8 genes are all localized on chromosome 12p12. The only other mammalian OATP promoter that has been characterized to date is the human OATP-A (SLC21A3) promoter (30). Unlike OATP-C, the OATP-A gene does not contain an intronic sequence within its 5′-untranslated region, and the promoter is functional in both hepatocyte-derived and nonhepatic cell lines such as CHO-K1 and MDCK. Based on sequence analysis and in accordance with its low expression level in the liver (23), no binding site for HNF1α is contained in the immediate 5′-flank-

The importance of HNF1α in directing liver-specific gene expression has become evident from the observation that numerous liver-specific genes possess HNF1 binding sites in their promoter or enhancer regions (14, 20). These include albumin, α1-antitrypsin (14), cytochromes P450 2E1 (21), and 7A1 (22) and the basolateral bile salt transporter Ntcp (12). HNF1α is a homeodomain-containing transcription factor that is enriched in liver but is also expressed in kidney, intestine, stomach, and pancreas (15, 17, 24). The tissue distribution of HNF1α indicates that liver specificity of gene expression cannot be accomplished by HNF1α alone but rather by the concerted action of a selected group of liver-enriched proteins including the HNF1, HNF3, HNF4, and CCAAT-enhancer binding protein families of transcription factors (25, 26). The OATP-C promoter was found to contain consensus recognition sites for several of these factors (Fig. 1B), which in combination are the likely effectors of liver-specific OATP-C expression.

A variant form of HNF1 termed HNF1β or vHNF1 has the same DNA binding specificity as HNF1α. Whereas HNF1α functions mainly in terminally differentiated cells and has been implicated in the maintenance of a correct differentiation state (15, 17), HNF1β is essential much earlier during development and is a key factor in visceral endoderm differentiation (14, 19). The OATP-C-derived HNF1 sequence that binds HNF1α was shown not to bind HNF1β (Fig. 5B). HNF1β is expressed at only low levels in the liver and cannot fulfil the function of HNF1α in vivo, as suggested (i) by the severe phenotype of HNF1α null mice that exhibit marked liver enlargement, elevated plasma transaminases, hypercholesterolemia, and hyperbilirubinemia (15, 24, 27) and (ii) by the observation that Oatp4 mRNA levels are strongly decreased in mice with a disrupted HNF1α gene (11), but are normal in mice with a conditional liver-specific HNF1β inactivation.3

The genomic organization of the OATP-C (SLC21A6) gene is similar to that of human OATP-A (SLC21A3) and OATP8 (SLC21A8) (5) and of mouse Oatp2 (Slc21a5) and Oatp4 (Slc21a6) (28, 29). The human OATP-A, OATP-C, and OATP8 genes are all localized on chromosome 12p12. The only other mammalian OATP promoter that has been characterized to date is the human OATP-A (SLC21A3) promoter (30). Unlike OATP-C, the OATP-A gene does not contain an intronic sequence within its 5′-untranslated region, and the promoter is functional in both hepatocyte-derived and nonhepatic cell lines such as CHO-K1 and MDCK. Based on sequence analysis and in accordance with its low expression level in the liver (23), no binding site for HNF1α is contained in the immediate 5′-flank-

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**Fig. 8. Transactivation of the human OATP8 (SLC21A8) and mouse Oatp4 (Slc21a6) promoters by HNF1α.** HepG2 cells were transfected with the 8-120 (hOATP8) or m4-137 (mOatp4) promoter constructs or with the promoterless pGL3-Basic vector (Basic) together with either an HNF1α expression plasmid (H9251, black bars) or with the empty pBluescript vector as a control (pBS-K, gray bars). Coexpression of HNF1α led to a 3.5-fold increase in both human OATP8 and mouse Oatp4 promoter activity. Cotransfection of the pBluescript vector had no effect on reporter activity. Luciferase activity is shown as the ratio firefly/Renilla luciferase (see "Experimental Procedures"), and data represent mean of ± 1 S.D. of three transfection experiments.

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**Table III**

| Species | Transporter | Gene symbol | Tissue distribution | Evidence for Regulation by HNF1α |
|---------|-------------|-------------|--------------------|---------------------------------|
| Human   | OATP-C      | SLC21A6     | Liver              | Promoter binds and is transactivated by HNF1α. |
|         | OATP8       | SLC21A8     | Liver              | Consensus binding site in promoter in the −47/−60 region; transactivation of promoter activity by coexpressed HNF1α in HepG2 cells. |
| Rat     | Ntcp        | Slc10a1     | Liver              | Promoter binds HNF1α; reduced nuclear HNF1α activity is associated with decreased Ntcp expression. |
| Mouse   | Ntcp        | Slc10a1     | Liver              | Reduced mRNA levels in the livers of HNF1α null (Tcf1−/−) mice. |
|         | Oatp1       | Slc21a1     | Liver, kidney      | Reduced mRNA levels in the livers of HNF1α null (Tcf1−/−) mice. |
|         | Oatp2       | Slc21a5     | Liver, brain       | Reduced mRNA levels in the livers of HNF1α null (Tcf1−/−) mice. |
|         | Oatp4       | Slc21a6     | Liver              | Consensus binding site in promoter in the −52/−66 region; transactivation of promoter activity by coexpressed HNF1α in HepG2 cells; reduced mRNA levels in the livers of HNF1α null (Tcf1−/−) mice. |

3 L. Gresh and M. Yaniv, unpublished observations.
ing region of the OATP-A gene (30).

In conclusion, this study provides evidence for the critical role of HNF1α in directing basal expression of the human OATP-C (SLC21A6) gene promoter. The finding that HNF1α binds to the OATP-C promoter and transactivates other liver-specific OATP promoters supports the notion that HNF1α is an essential factor in mediating hepatocyte-specific gene expression and in maintaining the differentiated hepatocellular phenotype. Perhaps most importantly, it lends support to the hypothesis that HNF1α is a global regulator of hepatic uptake of bile salts and bilirubin from systemic blood.

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Characterization of the Human OATP-C (SLC21A6) Gene Promoter and Regulation of Liver-specific OATP Genes by Hepatocyte Nuclear Factor 1 α

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