

Human Organic Anion Transporting Polypeptide 8 Promoter Is Transactivated by the Farnesoid X Receptor/Bile Acid Receptor

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Background & Aims: OATP8 (gene symbol: SLC21A8) is a multispecific uptake system for organic anions, xenobiotics, and peptides expressed at the basolateral (sinusoidal) membrane of human hepatocytes. We investigated whether OATP8 gene expression is regulated by the nuclear receptors farnesoid X receptor/bile acid receptor (FXR/BAR; NR1H4), pregnane X receptor (PXR), or liver X receptor (LXR). **Methods:** OATP8 promoter function was studied in reporter assays. OATP8 expression in cells was quantitated by real-time polymerase chain reaction. **Results:** The bile acid chenodeoxycholic acid (CDCA), a ligand of FXR/BAR, but not clotrimazole or 25-hydroxycholesterol, ligands of PXR or LXR, respectively, induced OATP8 promoter activity. An inverted hexanucleotide repeat motif (IR-1 element) in the promoter sequence was shown by electrophoretic mobility shift assays to bind the FXR (9-cis-retinoic acid receptor [RXR α]) heterodimer. Targeted mutagenesis of the IR-1 element abolished inducibility of the OATP8 promoter by CDCA, confirming its role as a bile acid response element. CDCA treatment increased OATP8 messenger RNA levels in human hepatoma cells, suggesting a physiologic role for FXR-mediated OATP8 gene regulation. **Conclusions:** OATP8 gene expression is regulated by bile acids via FXR/BAR. Induction of OATP8 could serve to maintain hepatic extraction of xenobiotics and peptides in conditions of increased intracellular bile acids.

A key function of the liver is the elimination of xenobiotics and endogenous catabolites from the systemic circulation. After uptake into hepatocytes, many of these compounds are biotransformed in phase I and phase II reactions and subsequently excreted into bile. Bile formation is driven primarily by the vectorial secretion of bile acids from blood into bile. Bile acids are efficiently retained within the enterohepatic circulation by active absorption in the ileum and extraction from portal blood by hepatocytes. The major hepatocellular uptake system for bile acids is the Na⁺-taurocholate

cotransporting polypeptide (NTCP). Na⁺-independent uptake is mediated by the organic anion transporting polypeptides (OATPs).^{1–5} OATP8 (solute carrier gene family [SLC]21A8) is a 702 amino acid protein that is expressed at the basolateral (sinusoidal) membrane of pericentral hepatocytes.⁴ Its preferred substrates include selected oligopeptides such as the opioid peptide [D-penicillamine^{2,5}]enkephalin and the gastrointestinal peptide hormone cholecystokinin,⁶ conjugated steroid metabolites such as estradiol-17 β -D-glucuronide and dehydroepiandrosterone-3-sulfate,⁴ and xenobiotics such as the cardiac glycoside digoxin.⁵ Low-efficiency transport of bile acids has also been shown.⁵

The aim of this study was to characterize the promoter region of the human OATP8 gene to gain initial insights into the mechanisms regulating OATP8 expression. We previously showed that the basis of liver-restricted expression of the liver-specific subfamily of OATPs, which includes human OATP-C (SLC21A6), human OATP8 (SLC21A8), and mouse Oatp4 (Slc21a6), is the dependence on hepatocyte nuclear factor (HNF)1 α binding to the minimal promoter region.⁷ Accordingly, an OATP8 promoter construct was shown to be transactivated by exogenous expression of HNF1 α in human hepatoblas-

Abbreviations used in this paper: BAR, bile acid receptor; BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DMSO, dimethyl sulfoxide; FXR, farnesoid X receptor; HepG2, human hepatoblastoma; HNF, hepatocyte nuclear factor; Huh7, human hepatoma; LCA, lithocholic acid; LMH, chicken hepatoma; NR1, orphan nuclear receptor; NTCP, Na⁺-taurocholate cotransporting polypeptide; Oatp/OATP, rodent/human organic anion transporting polypeptide; PCR, polymerase chain reaction; PXR, pregnane X receptor; RACE, rapid amplification of complementary DNA ends; RXR, retinoid X receptor; Slc/SLC, rodent/human solute carrier gene family; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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toma (HepG2) cells.⁷ In this study we identify a binding site for the farnesoid X receptor/bile acid receptor (FXR/BAR) in the OATP8 promoter. FXR/BAR is a major regulator of genes involved in bile acid transport and metabolism^{8–10} and its natural ligands are bile acids.^{11–13} Although HNF1 α appears to be a global regulator of the basolateral hepatocellular bile acid uptake systems NTCP and OATPs, but also of the FXR isoform FXR-1, it has no direct effect on the canalicular bile salt export pump (BSEP), the expression of which is unaltered in HNF1 α knockout (Tcf1^{-/-}) mice.¹⁴ However, BSEP expression is reduced in FXR^{-/-} mice, which is in agreement with the recent demonstration of FXR binding to the human BSEP gene promoter.¹⁵

We show here that the human OATP8 gene promoter also binds FXR and is transactivated by bile acids. This adds a level of complexity to the established notion that bile acids induce their own efflux into bile via BSEP but shut off the basolateral uptake systems such as NTCP¹⁶ in conditions associated with an increased bile acid load within hepatocytes. We propose a physiologic role for OATP8 gene induction by bile acids in light of current evidence for the pivotal role of FXR in governing bile acid transport and metabolism.

Materials and Methods

Materials

[γ -³²P] adenosine triphosphate (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Restriction enzymes and proteinase K were from Roche Molecular Biochemicals (Rotkreuz, Switzerland), Pfu-Turbo DNA polymerase from GibcoBRL (Basel, Switzerland), and T4 polynucleotide kinase from Stratagene (Amsterdam, Netherlands). Polyacrylamide was purchased from BioRad (Glattbrugg, Switzerland). Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Chemical Co. (Buchs, Switzerland).

Localization of the 5'-Region of the OATP8 Gene

Total RNA was isolated from human liver by the acid guanidinium/phenol/chloroform procedure.¹⁷ The 5'-end of the OATP8 messenger RNA (mRNA) was determined by using rapid amplification of complementary DNA ends (5'-rapid amplification of complementary DNA ends [RACE] system; Roche Molecular Biochemicals). A total of 1 μ g total RNA was reverse transcribed by using the OATP8-specific primer 8-RT (Table 1). The 5'-end was subsequently amplified by polymerase chain reaction (PCR) by using primer 8-PCRace and nested primer n8-PCRace as downstream primers (Table 1). The resulting 5'-RACE product was subcloned into pCRII-TOPO (Invitrogen BV, Groningen, Netherlands)

Table 1. Oligonucleotides Used for 5'-RACE, Chimeric Plasmid Construction, and Mobility Shift Assays

Oligonucleotide	Sequence (5' to 3')
8-RT	ACATAGATCCACATGTGTGACCCAG
8-PCRace	CCCATAAGGAGACAACCAATTC
n8-PCRace	TCTCTGAAGATGCTGACTCTGCTG
p-865	GCAACTGTATCAAGAGCTCTCTGTCACTG
p-438	CCATGTGAGCTCTCCAGTGTC
p-120	GATAGGCTTCTGGGGTGAGCTCCTAG
p-44	CATCATTGAGCTCATAAAAC
p+38	GAATGCTACAAGATCTGCAACAAGTCCATC
IR-1-TK-LUC	TAGGACAATGACCTAATAATGCCTAGGACAATGACCTA
wtlR-1	GTTTGCCTAGGACAATGACCTAATAAGAT
mutIR-1	GTTTGCCTAG TGCAATGA ACCAATAAGAT
8-for	CTGGAAGTATTTTGACATCTTTACC
8-rev	TGTTCCGTTGAATGATAAGGTTTG

NOTE. Mutated residues in the IR-1 element are indicated in bold.

and sequenced on an AlfExpress Sequencer (Amersham Pharmacia Biotech).

Plasmid Construction

Four fragments of the 5'-region of the OATP8 gene were PCR amplified by using human genomic DNA as a template, upstream primers p-865, p-438, p-120, or p-44, downstream primer p+38 (Table 1), and PfuTurbo DNA polymerase. The upstream primers contained an internal *SacI* restriction site, the downstream primer an internal *BglII* site. The resulting PCR products were digested with *BglII* and *SacI* and ligated into the luciferase reporter gene vector pGL3-Basic (Promega Catalys AG, Wallisellen, Switzerland) that had been predigested with *BglII* and *SacI*, yielding the following promoter constructs: LUC-865, LUC-438, LUC-120, and LUC-44. The IR-1-TK-LUC plasmid was constructed by ligating a dimerized oligonucleotide (IR-1-TK-LUC in Table 1) containing a tandem repeat of the IR-1 element of the OATP8 gene and 5'-*HindIII* and 3'-*BamHI* overhangs into TK-LUC plasmid predigested with *HindIII* and *BamHI*. Sequence identity of all constructs with the OATP8 gene was verified by sequence analysis. Plasmid DNA was prepared by using the Qiagen system (Basel, Switzerland).

Site-Directed Mutagenesis

An OATP8-derived -120/+38 construct containing staggered nicks was generated by PCR by using 2 complementary oligonucleotides mutated in the IR-1 binding site (sequence mutIR-1 in Table 1) and PfuTurbo DNA polymerase (GibcoBRL). The product was digested with *DpnI* to remove the parental DNA template and select for DNA containing the mutation. The mutated plasmid was termed *mutIR-1*.

Culture and Transfection of Chicken Hepatoma Cells

Chicken hepatoma (LMH) cells were obtained from ATCC (Rockville, MD) and were grown in William's Medium

E (GibcoBRL) supplemented with 10% fetal calf serum (GibcoBRL), 2 mmol/L glutamine (GibcoBRL), 1 × nonessential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (GibcoBRL), on gelatin-coated dishes (Sigma). Thirty-six hours before transfection, cells were seeded on to gelatin-coated 24-well plates in medium supplemented with 10% charcoal stripped bovine calf serum (Sigma) at 75%–80% density. Transfections were performed with 1.5 µL FuGENE 6 transfection reagent (Roche Molecular Biochemicals) and 500 ng plasmid DNA, the latter comprising 450 ng luciferase construct and 50 ng pSV-β-galactosidase plasmid (Promega Catalys AG). Six hours after transfection, cells were stimulated with bile acids or dimethyl sulfoxide (DMSO) for 24 hours.

Culture and Transfection of Human Cell Lines

HepG2 and human hepatoma (Huh7) cell lines were purchased from ATCC. Cells were maintained in RPMI1640 (Sigma) supplemented with 10% fetal calf serum (GibcoBRL), 100 U/mL penicillin, and 100 µg/mL streptomycin (GibcoBRL). For transactivation assays or RNA preparation, cells were grown for 3 days in medium containing 10% charcoal-stripped bovine calf serum and then seeded at 90%–95% density in 24-well plates (reporter assays) or 6-well plates (RNA isolation). For transient transfections, 1.5 µL of Lipofectamin 2000 reagent (GibcoBRL) and 500 ng of plasmid DNA were used per well. For reporter assays, plasmid DNA comprised 350 ng LUC-120 promoter construct, 50 ng pSV-β-galactosidase plasmid, and 50 ng each of pCMX-human retinoid X receptor (RXR)α and pCMX-hFXR expression plasmid. For RNA preparation cells were transfected with 250 ng of pCMX-hRXRα and pCMX-hFXR expression plasmid. To ensure that the total DNA amount transfected remained constant, pBluescript vector (pB-SKII, Clontech, Basel, Switzerland) was used as carrier DNA as required. Twenty-four hours after transfection, cells were treated with bile acids as indicated or DMSO for 24 hours.

Luciferase and β-Galactosidase Reporter Assays

Cells were lysed with passive lysis buffer (PLB; Promega Catalys AG) 24 hours after DMSO or bile acid treatment. Luciferase activity was quantified by using the luciferase assay system (Promega Catalys AG) in a Lumat LB 9507-2 luminometer (Berthold, Bad Wildbad, Germany). β-galactosidase activity was quantified with a high-sensitivity assay (Stratagene) in a UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 595 nm.

Quantitation of OATP8 Gene Expression

A total of 1 µg total RNA isolated from Huh7 or HepG2 cells was reverse transcribed (Reverse Transcription System; Promega Catalys AG). Real-time PCR was performed with the ABI PRISM 7700 sequence detection system using one sixth of the RT reaction and was analyzed with the 1.7

software (Applied Biosystems, Rotkreuz, Switzerland). Amplification of the endogenous control was performed with the ribosomal 18S TaqMan PCR master system (Applied Biosystems). OATP8 was amplified with the primer 8-for and 8-rev (Table 1) by using cyber green incorporation (SYBR Green PCR-Master Mix; Qiagen). Because validation experiments showed that amplification efficiencies of the target and the reference were approximately equal, quantitation was performed with the comparative $\Delta\Delta C_T$ method.

Electrophoretic Mobility Shift Assays

Double-stranded oligonucleotide probes were obtained by hybridizing single-stranded complementary oligonucleotides (Microsynth, Balgach, Switzerland). Dimers with the wtIR-1 sequence corresponding to the sequence found in the OATP8 gene promoter (Table 1) were labeled with [γ - 32 P]adenosine triphosphate by using T4 polynucleotide kinase (Stratagene). Nuclear extracts were prepared as described.¹⁸ In vitro translation was performed with the TnT Quick coupled transcription/translation system (Promega Catalys AG).

For gel mobility shift assays, 5 µL of in vitro translated FXR or RXRα protein or 5 µg of nuclear extracts were incubated on ice for 20 minutes with 2–5 fmol [γ - 32 P]-end-labeled dimerized oligonucleotide and 1 µg poly(dI)poly(dC) (Amersham Pharmacia Biotech) in 20 mmol/L HEPES-KOH, pH 7.9, 20% glycerol, 100 mmol/L KCl, 2 mmol/L MgCl₂, 0.5 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride. For competition assays, a 10- to 500-fold excess of unlabeled dimerized oligonucleotides was added. Sequence mutIR-1 (Table 1) corresponded to the wild-type OATP8 sequence mutated within the IR-1 site. For supershift experiments, 2 µL antibody against FXR (C-20: sc-1204, 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 hours.

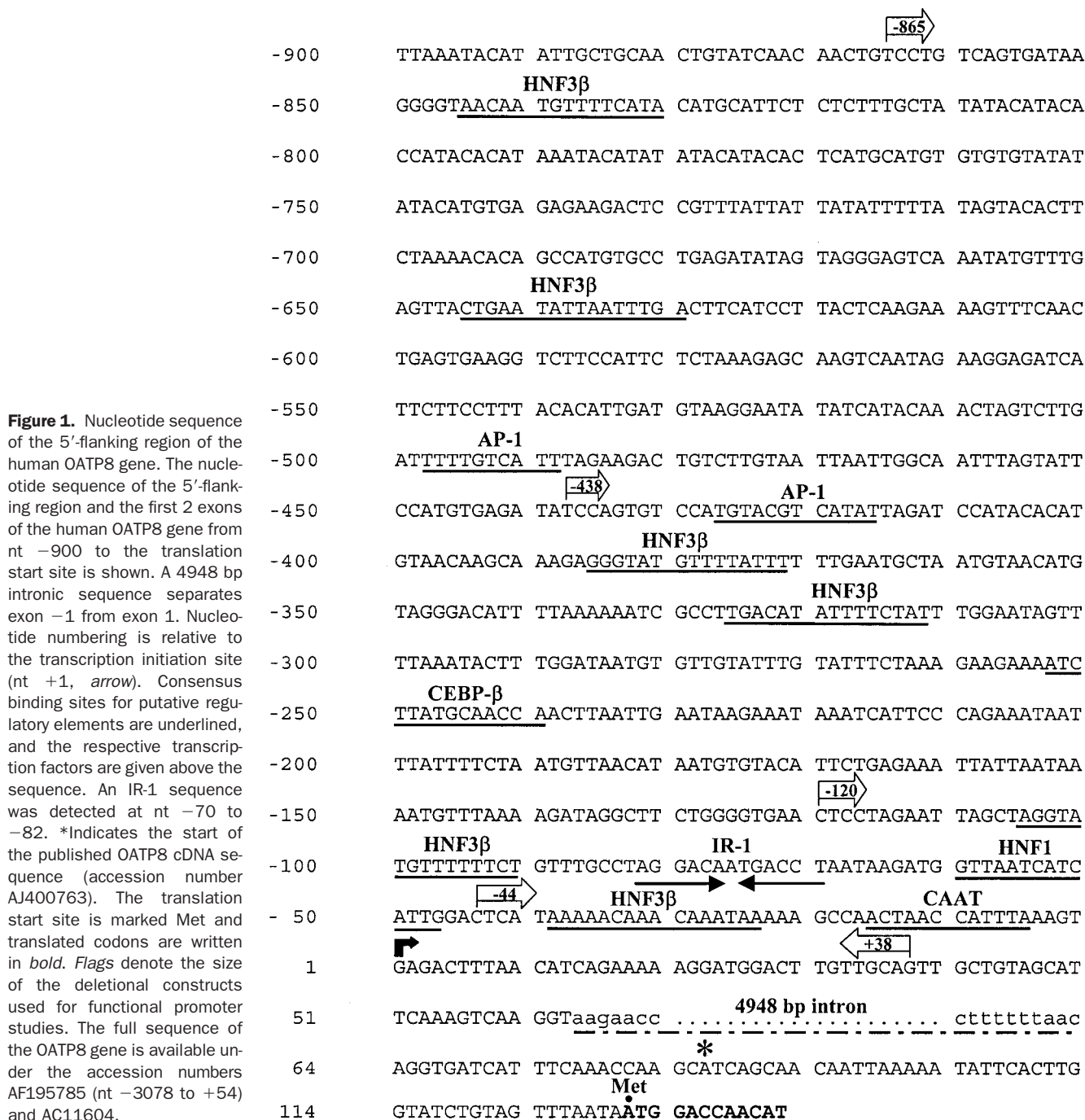
Statistical Analysis

Reporter gene activities are expressed as the mean ± 1 SD of at least 3 individual transfection experiments. All data were reproduced at least once by using 2 different preparations of plasmid DNA.

Results

Localization of the Transcription Initiation Site and Promoter Region of the OATP8 (SLC21A8) Gene

To localize the promoter region of the OATP8 gene, the transcription initiation site was identified by a 5'-RACE approach. By using a downstream oligonucleotide (n8-PCRace in Table 1) that corresponded to nucleotides 96-73 of the OATP8 cDNA sequence as published by König et al.⁴ (accession number AJ251506), a



single PCR product was amplified from human liver mRNA. Compared with the published cDNA sequence,⁴ 22 additional nucleotides are present in the 5'-untranslated region of exon 1. These additional nucleotides are also present in the genomic SLC21A8 sequence derived from BAC clone RP11-80N2 (AC011604). Based on the genomic SLC21A8 sequence, an additional exon (exon -1) with a length of 63 bp is separated by a 4948 bp intronic sequence from the 67 bp untranslated region of exon 1. The first nucleotide of exon -1 was designated

as the transcription start site (nt +1 in Figure 1). The translation start site is localized at nt 68 of exon 1. A similar genomic structure without sequence information was reported by Abe et al.¹⁹

Analysis of the 5'-Flanking Region of the Human OATP8 (SLC21A8) Gene

The 5'-flanking region of the OATP8 gene was PCR amplified from human genomic DNA and showed sequence identity with clone Homo sapiens 12p12-31.7-

37.2 BAC RP11-80N2 (AC011604). Figure 1 displays the sequence of the 5'-flanking 900 nt relative to the transcription initiation site up to the translation start site. Potential transcription factor recognition sites were identified by using the program Mat Inspector (Genomatix Software, Munich, Germany) and included more generally represented DNA elements such as the activator protein 1 at nt -416 to -427 and -489 to -498 and a CAAT element at -5 to -16, as well as liver-enriched transcription factor binding sites (e.g., for HNF1 at -47 to -60, CCAAT-enhancer binding protein- β at -240 to -253, and several potential HNF3 β binding sites, Figure 1).

By using a weighted matrix-based computational approach, we identified a nuclear receptor binding site arranged as an inverted hexanucleotide repeat separated by a single base (AGGACAaTGACCT) at position -70 to -82 of the OATP8 promoter sequence. Nuclear receptor DNA recognition sites contain consensus hexameric repeat motifs (AGAACA or AGGTCA) that can be organized as direct, everted, or inverted repeats and are spaced by a defined number of nucleotides.^{20,21} The IR-1 motif present in the OATP8 promoter has been shown to bind the FXR/RXR α heterodimer^{13,22-24} and to serve as a bile acid response element.^{15,25,26} The primary ligand for mouse, rat, and human FXR is the bile acid chenodeoxycholic acid (CDCA).¹¹⁻¹³

Effects of Bile Acids on OATP8 Promoter Activity in LMH Cells

To investigate whether the IR-1 motif in the OATP8 gene promoter is a functional bile acid response element, we initially transfected LMH cells with 4 OATP8 luciferase constructs and with the promoterless pGL3-Basic luciferase vector. LMH cells have been used extensively as a model for ligand-dependent activation of endogenously expressed nuclear receptors²⁷⁻²⁹ and have maintained the molecular induction mechanisms characteristic of mammalian liver.²⁷ The amino acid identity between chicken and human FXR is 95.5% in the DNA-binding domain and 86% in the ligand-binding domain and CDCA is the most potent activator of chicken FXR (M. Podvinec and U. A. Meyer, unpublished observations, 2001). LMH cells thus seemed suitable to study ligand-dependent activation of OATP8 promoter function by FXR. OATP8 promoter activity in LMH cells was assayed in the presence or absence of the FXR ligand CDCA. In the absence of CDCA, a 7.5- (LUC-865 construct) to 10.6-fold (LUC-438) induction of reporter gene activity was observed compared with the promoterless pGL3-Basic plasmid (Figure 2A). The LUC-44

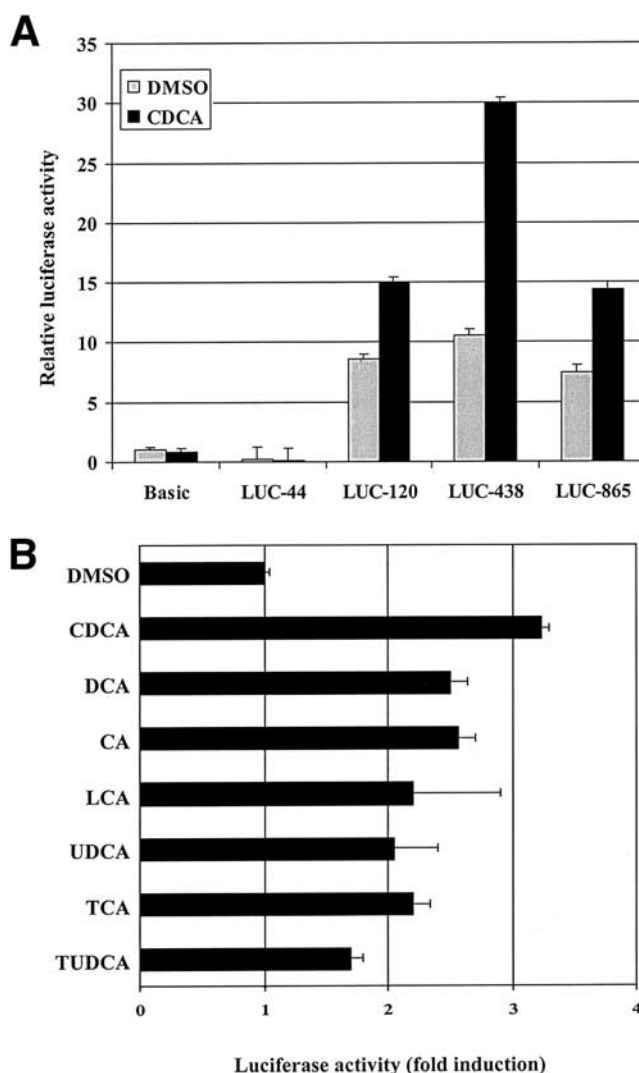


Figure 2. Analysis of OATP8 promoter function in LMH cells and transactivation by bile acids. (A) LMH cells were transiently transfected with 4 chimeric reporter gene constructs containing from 865 (LUC-865) to 44 (LUC-44) nucleotides of 5'-flanking sequence and extending to nt +38. Promoter activity is expressed in relation to background activity measured in cells transfected with pGL3-Basic alone (Basic). Promoter activity in cells treated with CDCA (100 μ mol/L) over 24 hours was compared with that measured in DMSO-treated cells. CDCA led to a 2-3-fold induction of all OATP8 promoter constructs with the exception of LUC-44, which lacked promoter activity. \square , DMSO; \blacksquare , CDCA. (B) LMH cells were transiently transfected with the LUC-120 construct and treated with DMSO or various bile acids (CDCA 50 μ mol/L, DCA 100 μ mol/L, CA 100 μ mol/L, LCA 50 μ mol/L, UDCA 100 μ mol/L, TCA 100 μ mol/L, TUDCA 100 μ mol/L). Transfection efficiency was normalized by cotransfection of pSV- β -galactosidase, and promoter activity is expressed as relative light units luciferase per unit of β -galactosidase (see Materials and Methods section). Data represent the mean \pm 1 SD of 3-5 transfections.

construct (containing nt +38 to -44) did not confer any luciferase activity, indicating that factors that bind within the -45 to -120 region, such as HNF1 α ,⁷ are required for minimal promoter activity. In the presence of 50 μ mol/L CDCA, the induction of luciferase activity

over the pGL3-Basic vector was 14.4- (LUC-865) to 30-fold (LUC-438), indicating a 2- to 3-fold increase in promoter activity secondary to CDCA treatment (Figure 2A). The CDCA-induced increase in promoter activity was also observed for the LUC-120 construct, indicating that the response element was located within the +38/-120 region (Figure 2A).

To compare the potency of different bile acids in activating the OATP8 promoter, LMH cells were transfected with the LUC-120 construct and were incubated with various bile acids at the following concentrations (in $\mu\text{mol/L}$): CDCA 50, deoxycholic acid (DCA) 100, cholic acid (CA) 100, lithocholic acid (LCA) 50, ursodeoxycholic acid (UDCA) 100, taurocholic acid (TCA) 100, tauroursodeoxycholic acid (TUDCA) 100. Compared with DMSO-treated controls, all bile acids led to a 1.7-fold (TUDCA) to 3.2-fold (CDCA) induction of OATP8 gene transcriptional activity (Figure 2B). To exclude that the OATP8 promoter fragment is induced by nuclear receptors other than FXR, LMH cells transfected with the LUC-865 construct were treated with known ligands for the chicken xenobiotic receptor and pregnane X receptor (PXR) (10 $\mu\text{mol/L}$ clotrimazole),^{27,28,30} and the liver X receptor (LXR) (10 $\mu\text{mol/L}$ 25-hydroxycholesterol, 10 $\mu\text{mol/L}$ 22(S)-hydroxycholesterol).^{31,32} Neither PXR nor LXR ligands had a detectable effect on OATP8 promoter activity (data not shown).

The IR-1 Binding Site Confers Activation by CDCA

To assess the importance of the IR-1 element for the bile acid dependent activation of the OATP8 gene, a tandem repeat of the IR-1 element was cloned in front of the thymidine kinase promoter of the luciferase reporter gene vector TK-LUC (Figure 3A). The resulting reporter plasmid IR-1-TK-LUC contained 2 copies of the IR-1 binding site as present in the sequence of the OATP8 gene. In transfected LMH cells, both the native TK-LUC and the modified IR-1-TK-LUC plasmid had similar basal luciferase activities attributable to the TK promoter (Figure 3A). Treatment with CDCA (50 $\mu\text{mol/L}$) resulted in a 10.8-fold induction of IR-1-TK-LUC, whereas native TK-LUC showed no response to CDCA. To further confirm the role of the IR-1 motif in mediating the induction by CDCA, mutations were introduced into the IR-1 sequence of the -120/+38 construct by site-directed mutagenesis. The resulting reporter plasmid mutIR-1 contained mutations in the -82/-70 region as shown in Figure 3B. CDCA-dependent induction of the OATP8 promoter was completely abol-

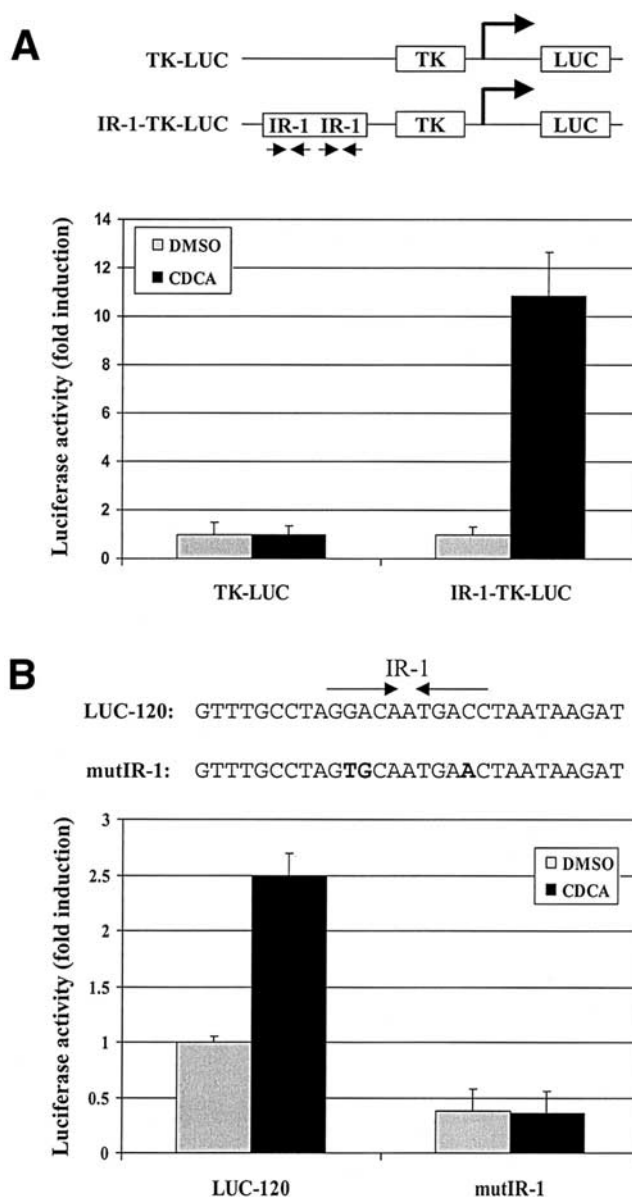


Figure 3. Role of the IR-1 binding site in CDCA induction of the OATP8 promoter in LMH cells. □, DMSO; ■, CDCA. (A) CDCA induction of a tandem repeat of the IR-1 motif in a heterologous promoter context. LMH cells were transfected with either the TK-LUC plasmid or the IR-1-TK-LUC plasmid that contained 2 IR-1 elements in front of the TK promoter. Treatment with CDCA (50 $\mu\text{mol/L}$) resulted in a 10.8-fold induction of IR-1-TK-LUC, whereas TK-LUC-associated luciferase activity was unaffected. Promoter activity is shown as the ratio luciferase/ β -galactosidase, and data represent the mean \pm 1 SD of 3–5 transfections. (B) Site-directed mutagenesis of the IR-1 element. LMH cells were transfected with either the LUC-120 construct containing the wild-type OATP8 sequence, or the mutIR-1 construct containing targeted mutations within the IR-1 element. Mutation of the IR-1 site resulted in reduced overall promoter activity and a complete loss of inducibility by CDCA (50 $\mu\text{mol/L}$).

ished in mutIR-1-transfected LMH cells (Figure 3B). Furthermore, basal promoter activity in the absence of CDCA was also significantly reduced compared with the LUC-120 construct. These data confirmed the

functional role of the IR-1 motif as a bile acid response element.

FXR/RXR α Transactivates the OATP8 Promoter in HepG2 Cells

To confirm that transactivation of the OATP8 promoter by bile acids is dependent on the FXR/RXR α heterodimer, the effect of CDCA on LUC-120-mediated luciferase activity was studied in HepG2 cells transfected with the human FXR/RXR α expression plasmids compared with cells transfected with pB-SK alone. Unlike LMH cells, HepG2 cells have low endogenous FXR and RXR α expression levels^{15,16} and the presence of 100 μ mol/L CDCA had no effect on OATP8 promoter activity (pB-SK in Figure 4A). Cotransfection of human FXR or RXR α expression plasmid alone led to a 2- to 3-fold increase in basal promoter activity, but not to inducibility by CDCA. In contrast, cotransfection of FXR and RXR α in combination increased promoter activity 2.8-fold in the absence and 5.8-fold in the presence of 100 μ mol/L CDCA (Figure 4A), indicating that the bile acid response in the reporter assay is dependent on exogenous expression of FXR/RXR α . The 2.8-fold increase in basal activity in the absence of CDCA may have been caused by endogenously synthesized bile acids in HepG2 cells,³³ which presumably represent ligands for FXR.

We next compared the ability of different bile acids to transactivate the LUC-120 promoter construct in FXR/RXR α -cotransfected HepG2 cells. With reference to luciferase activity in DMSO-treated controls, a significant increase in promoter activity was induced by the hydrophobic bile acids CDCA (2.1-fold), DCA (1.7-fold), and LCA (1.4-fold) (Figure 4B). In contrast to transactivation assays in LMH cells (Figure 2B), the more hydrophilic bile acids CA, TCA, UDCA, and TUDCA had no effect on OATP8 promoter activity in HepG2 cells. A similar induction pattern by bile acids was observed in FXR/RXR α -transfected Huh7 cells (data not shown).

The IR-1 Element in the OATP8 Gene Binds the FXR/RXR α Heterodimer

To determine whether the FXR/RXR α heterodimer indeed binds to the IR-1 element in the OATP8 promoter, electrophoretic mobility shift assays were performed. A ³²P-labeled dimerized oligonucleotide corresponding to nt -62 to -90 of the OATP8 gene (wtIR-1 in Table 1) was incubated with *in vitro* translated monomeric FXR or RXR α or with the FXR/RXR α heterodimer. Although monomeric RXR α protein was not able to bind to the IR-1 element, incubation with monomeric FXR resulted in a DNA-protein com-

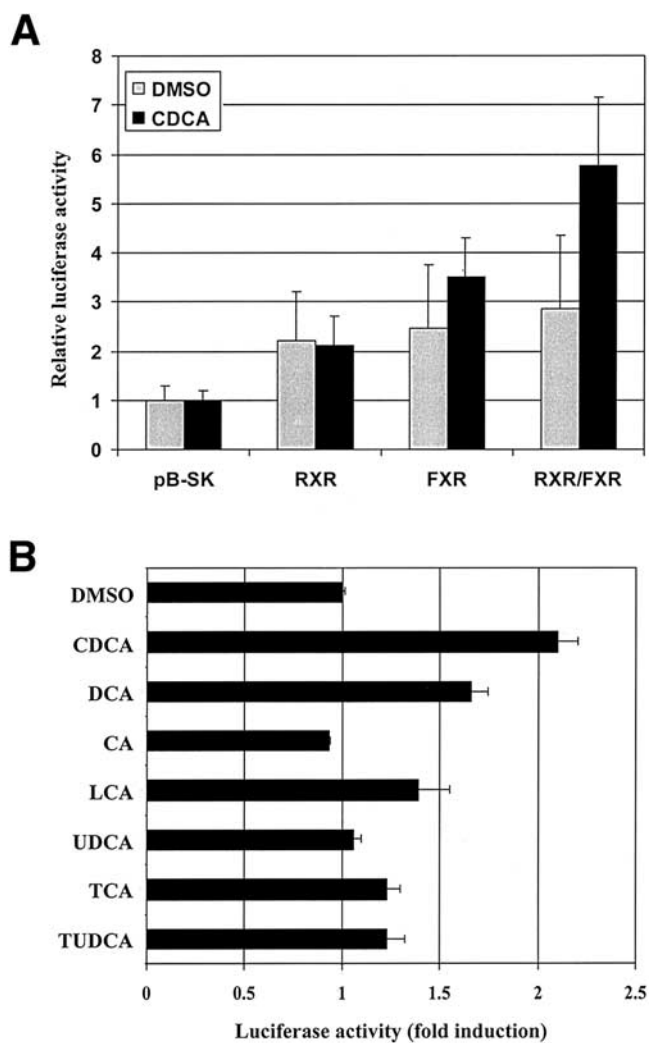


Figure 4. Effect of exogenous FXR and RXR α overexpression on OATP8 promoter function in HepG2 cells. (A) HepG2 cells were transfected with the LUC-120 construct together with FXR and RXR α expression plasmids alone or in combination, or with carrier DNA alone (pB-SK). Twenty-four hours after transfection, cells were treated with 100 μ mol/L CDCA or with DMSO for 24 hours. Promoter activity is expressed in relation to measurements in DMSO-treated cells transfected with LUC-120 and pB-SK alone. \square , DMSO; \blacksquare , CDCA. (B) HepG2 cells were transiently transfected with LUC-120 and the FXR/RXR α expression plasmids. Cells were treated with DMSO or various bile acids (CDCA 100 μ mol/L, DCA 100 μ mol/L, CA 100 μ mol/L, LCA 50 μ mol/L, UDCA 100 μ mol/L, TCA 100 μ mol/L, TUDCA 100 μ mol/L). Promoter activity is shown as the ratio luciferase/ β -galactosidase, and data represent the mean \pm 1 SD of 3–5 transfections.

plex (lower band in Figure 5). The addition of FXR and RXR α in combination to the labeled IR-1 element resulted in formation of 2 DNA-protein complexes. The lower band exhibited the same electrophoretic migration pattern as the complex formed in the presence of FXR alone, suggesting binding of monomeric FXR to the IR-1 element. The upper band resulted from binding of the FXR/RXR α heterodimer. Addition of a specific antibody against FXR in the presence of the FXR and

<i>in vitro</i> translation						
RXR	+	-	+	+	+	+
FXR	-	+	+	+	+	+
Competitor					wt	mut
FXR Antibody	-	-	-	+	-	-

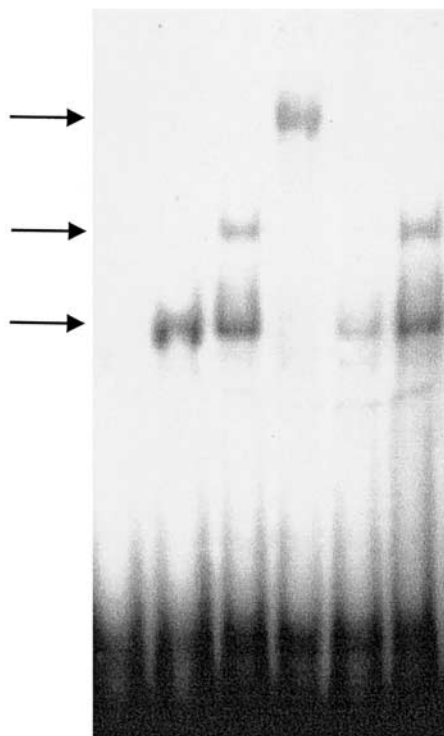


Figure 5. The IR-1 element in the OATP8 promoter binds FXR/RXR α . Electrophoretic mobility shift assays were performed by using a 32 P-labeled wtIR-1 -62/-90 oligonucleotide (Table 1) that contained the IR-1 binding site. Addition of *in vitro* translated RXR α did not produce a visible complex, whereas the addition of monomeric FXR resulted in a DNA-protein complex (*lower arrow*). Addition of FXR and RXR α in combination resulted in binding of heterodimeric FXR/RXR α (*middle arrow*). The addition of an antibody targeted against FXR resulted in a supershift (*top arrow*) and disappearance of the lower bands. Competition experiments using a 500-fold excess of unlabeled wtIR-1 oligonucleotide (wt) completely inhibited binding of FXR/RXR α to the 32 P-labeled wtFXR oligonucleotide, whereas a 500-fold excess of mutIR-1 oligonucleotide with a mutated IR-1 recognition site (Table 1) had no effect (mut).

RXR α proteins produced a single supershifted complex. Furthermore, DNA-protein complex formation was inhibited by the addition of an excess of unlabeled wtIR-1 oligonucleotide, but was unaffected by the presence of excess mutIR-1 oligonucleotide (Table 1) with a mutated IR-1 element (Figure 5). These results confirmed the specificity of binding of monomeric FXR and heterodimeric FXR/RXR α to the IR-1 motif in the OATP8 promoter.

To confirm that the IR-1 element also binds FXR/RXR α in LMH and HepG2 cells, electrophoretic mo-

bility shift assays were repeated by using nuclear extracts from these cell lines. Nuclear extracts from LMH cells produced a DNA protein complex that was not formed in the presence of excess unlabeled wtIR-1 oligonucleotide but was unaffected by the addition of mutated mutIR-1 oligonucleotide (lower arrow in Figure 6). The presence of antibody against FXR resulted in the formation of a supershifted complex, indicating the specificity of FXR/RXR α binding (upper arrow in Figure 6). Nuclear extracts from LMH cells that had been pretreated with CDCA (100 μ mol/L) for 24 hours produced the identical pattern and intensity of DNA-protein complex formation compared with cells treated with DMSO alone.

Incubation of the labeled wtIR-1 oligonucleotide with nuclear extracts prepared from FXR/RXR α cotransfected HepG2 cells also led to the formation of a DNA-protein complex. Complex formation was inhibited in the presence of excess unlabeled wtIR-1 but not mutIR-1 oligonucleotide (Figure 6). Addition of antibody against FXR resulted in competition of complex formation, confirming the specificity of FXR/RXR α binding to the IR-1. Nuclear extracts prepared from untransfected HepG2 cells produced the same DNA-protein complex, albeit of very weak intensity (data not shown). This strengthens the notion that HepG2 cells endogenously express FXR and RXR α at low levels.

The pattern of DNA-protein complex formation by using FXR/RXR α -transfected HepG2 cells that had been pretreated with CDCA before nuclear protein extraction showed no detectable difference compared with DMSO-treated cells (Figure 6), confirming the results obtained in LMH cells. The mechanism of CDCA activation of the OATP8 promoter is thus not attributable to a quantitative effect on FXR/RXR α binding to the IR-1 element but may involve recruitment of coactivator complexes³⁴ or conformational changes of its target receptor FXR.

Concentration Dependence of CDCA Activation of the OATP8 Gene

To investigate whether FXR-mediated activation of the OATP8 gene is only evident at high bile acid concentrations of 50–100 μ mol/L or also at lower, more physiologic, concentrations, LMH and HepG2 cells were transfected with the LUC-120 construct and were incubated with CDCA concentrations ranging from 0–200 μ mol/L. LMH cells showed a dose-dependent increase in OATP8 promoter activation up to CDCA concentrations of 25–50 μ mol/L, with weaker induction at 100 μ mol/L and an absence of induction at 200 μ mol/L (Figure 7A). Essentially

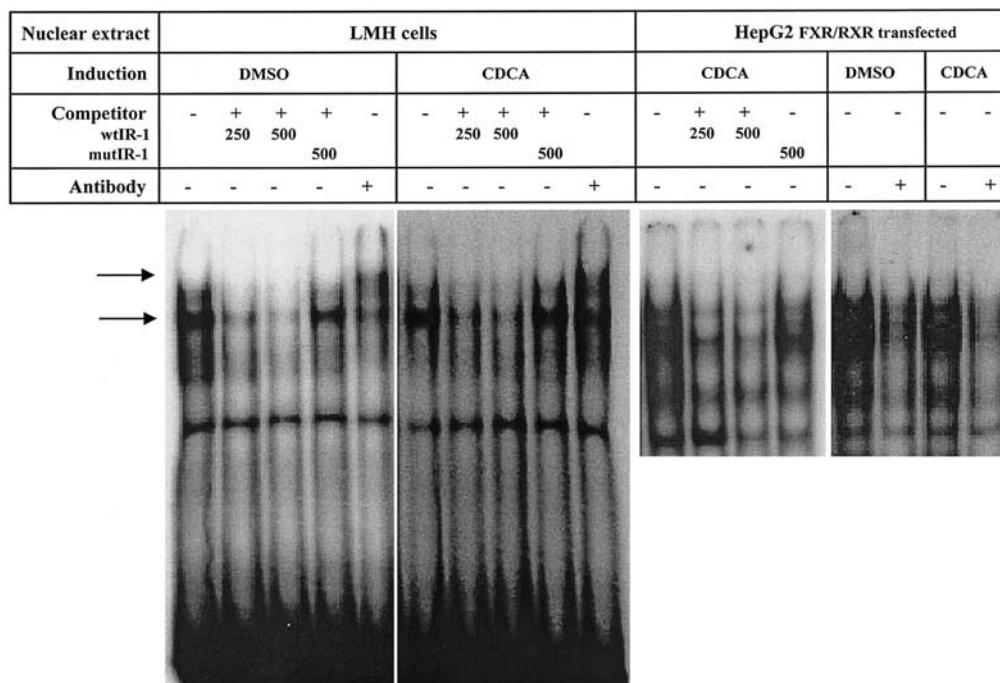


Figure 6. Binding of nuclear proteins from LMH and FXR/RXR α -transfected HepG2 cells to the IR-1 element in the OATP8 promoter. The left 2 panels show results obtained with nuclear extracts from LMH cells, the right 2 panels show FXR/RXR α -transfected HepG2 cells. A protein complex with the 32 P-labeled wtIR-1 oligonucleotide (Table 1) that resulted in a band shift (*lower arrow*) was formed in the presence of nuclear extracts from both LMH cells and FXR/RXR α -transfected HepG2 cells. Competition experiments using a 250- to 500-fold excess of unlabeled wtIR-1 oligonucleotide showed complete inhibition of complex formation (wtIR-1), whereas a 500-fold excess of mutIR-1 oligonucleotide with a mutated IR-1 recognition site (mutIR-1) had no effect. Antibody against FXR shifted the wtIR-1 bound protein complex in LMH cells (*top arrow*) or competed off complex formation in HepG2 cells. No differences in the pattern or intensity of DNA-protein complex formation were seen between DMSO-treated cells (DMSO) and cells treated with 100 μ mol/L CDCA (CDCA).

similar effects were found in FXR/RXR α -cotransfected HepG2 cells exposed to increasing concentrations of CDCA (Figure 7B). In contrast to LMH cells, a submaximal effect was already seen at 10 μ mol/L, with only minor additional increases up to 100 μ mol/L. Again, increasing the CDCA concentration to

200 μ mol/L practically abolished induction. The differences in concentration dependence between LMH and HepG2 cells could reflect differences in the affinities of chicken and human FXR for CDCA or differences in the efficiency of CDCA uptake by the respective cell line.

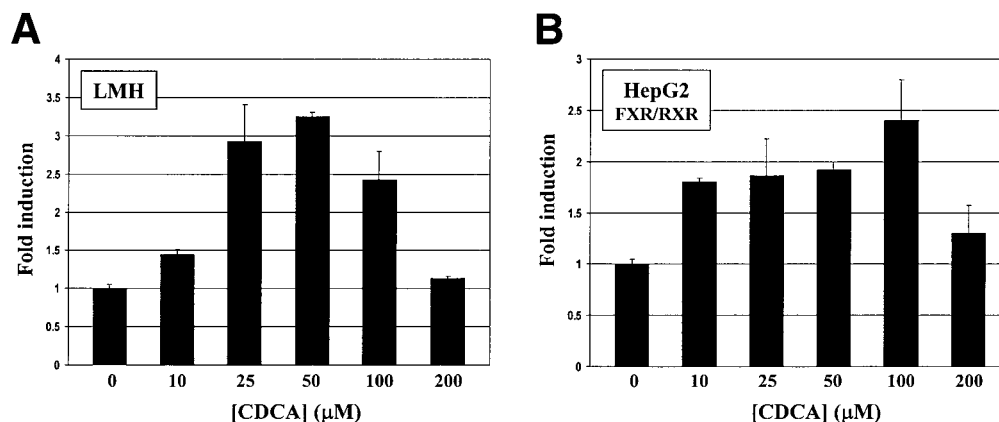


Figure 7. Concentration dependence of OATP8 promoter induction by CDCA. (A) LMH cells were transfected with the LUC-120 construct and treated with CDCA at concentrations ranging from 0–200 μ mol/L. (B) HepG2 cells were cotransfected with the LUC-120 construct and FXR/RXR α expression plasmids. Twenty-four hours after transfection, cells were treated with CDCA at concentrations ranging from 0–200 μ mol/L. Promoter activity is shown as the ratio luciferase/ β -galactosidase and is expressed in relation to measurements in cells treated with DMSO alone. Data represent the mean \pm 1 SD of 3–5 transfections.

Table 2. Induction of OATP8 Expression in Huh7 Cells by CDCA

Huh7 cells: treatment group	18S Average C _T	OATP8 Average C _T	ΔC _T OATP8-18S	ΔΔC _T ΔC _T -ΔC _T DMSO	Amount of OATP8 mRNA
Untransfected-DMSO	15.83 ± 0.17	49.9 ± 0.4	34.07 ± 0.43	0 ± 0.6	1.0
Untransfected-CDCA	16.2 ± 0.15	44.12 ± 0.44	27.92 ± 0.46	-6.15 ± 0.62	71.0
FXRα/RXRα-DMSO	17.11 ± 0.06	48.41 ± 0.14	31.1 ± 0.28	-2.77 ± 0.55	6.8
FXRα/RXRα-CDCA	17.3 ± 0.35	44.85 ± 1.72	27.55 ± 1.7	-6.52 ± 1.7	91.8

NOTE. Total RNA was isolated from untransfected Huh7 cells or Huh7 cells transfected with FXRα/RXRα expression plasmids. Cells were treated with DMSO as a control or with CDCA (100 μmol/L, 24 hours). A total of 1 μg RNA was reverse transcribed and used for relative quantitation of gene expression. Amplification of endogenous 18S ribosomal RNA was performed to standardize the amount of OATP8 mRNA. For relative quantitation, the comparative C_T method was used. C_T is the threshold cycle for target amplification, ΔC_T represents the difference in threshold cycles for target and reference. The amount of target (OATP8) mRNA, normalized for the endogenous reference and measured in relation to the calibrator (untransfected DMSO-treated cells), was calculated as 2^{-ΔΔC_T}.

Quantitation of Endogenous OATP8 Expression in Cell Lines

Having established that the OATP8 promoter binds FXR/RXR and is transactivated by CDCA, we tested whether endogenous OATP8 expression in human cell lines is increased in response to CDCA. FXRα/RXRα-transfected or untransfected Huh7 and HepG2 cells were treated with DMSO or 100 μmol/L CDCA. Subsequently, endogenous OATP8 mRNA levels were quantitated by real-time PCR. Because a validation experiment showed that amplification efficiencies of target (OATP8) and reference (18S RNA) were approximately equal, relative quantitation was performed with the comparative ΔΔC_T method.^{35,36} CDCA treatment of untransfected Huh7 cells resulted in a 71-fold increase in the amount of OATP8 mRNA compared with DMSO-treated control cells (Table 2). Transfection of Huh7 cells with FXRα and RXRα produced a 6.8-fold increase in OATP8 mRNA in the absence of CDCA, presumably owing to activation by endogenously synthesized bile acids.³³ CDCA treatment of FXRα/RXRα-transfected Huh7 cells led to a 91.7-fold increase in the amount of OATP8 mRNA compared with untransfected DMSO-treated cells (Table 2). A similar pattern of induction of OATP8 gene expression by CDCA treatment was observed in HepG2 cells (data not shown). These data showed that binding of FXR/RXR to the OATP8 promoter is physiologically relevant and confers significant induction of endogenous OATP8 mRNA levels in human cell lines by the FXR ligand CDCA.

Discussion

This study reports the characterization of the promoter region of the human OATP8 (SLC21A8) gene and the identification of a binding site for the farnesoid X receptor (bile acid response element). The region from nt -70 to -82 relative to the transcription initiation

site contains an inverted hexanucleotide repeat, a so-called IR-1 element, with hexameric repeat motifs characteristic of the binding sites for nuclear receptors (Figure 1).^{20,21} The IR-1 motif has been shown to bind the nuclear receptor FXR/BAR, also known as NR1H4, HRR1, and RXR-interacting protein 14.^{22,37} FXR is expressed in liver, intestine, kidney, and adrenal cortex, tissues in which bile acids are present.²² Similar to other members of the orphan nuclear receptor (NR1) family, FXR binds DNA as a heterodimer with the 9-cis-retinoic acid receptor RXR.³⁸ The natural ligands of FXR are bile acids, and CDCA, LCA, and DCA (as well as their taurine and glycine conjugates), have been shown by ligand-binding assays to directly bind FXR.^{11,12} The physiologic role of FXR as a bile acid receptor has been unequivocally established.^{11-13,24}

To determine whether the IR-1 element in the OATP8 gene promoter confers inducibility by bile acids, chicken hepatoma LMH cells, known to endogenously express nuclear receptors at sufficient levels to confer induction of target genes by the appropriate ligands,²⁷⁻²⁹ were transfected with chimeric OATP8 promoter constructs. All tested bile acids induced OATP8 promoter activity with the strongest effect exerted by CDCA (Figure 2), which is in agreement with results from ligand-binding assays indicating strongest activation of FXR by the hydrophobic bile acids CDCA, DCA, and LCA.^{11,12,24} The taurine conjugates of CA and UDCA also led to OATP8 promoter activation, albeit to a lesser degree (Figure 2). A tandem repeat of the IR-1 motif strongly enhanced reporter gene activity from a thymidine kinase promoter luciferase construct in the presence of CDCA (Figure 3A). Conversely, site-directed mutagenesis of the IR-1 element abolished the bile acid response in LMH cells (Figure 3B), confirming the functional significance of the IR-1 as a bile acid response element.

To investigate the effect of exogenously coexpressed FXR and RXR α on promoter function, HepG2 cells were used as a human hepatocyte-derived cell line with low endogenous expression of FXR and RXR α .¹⁵ In the absence of coexpressed FXR/RXR α , promoter activity was unaffected by exposure of HepG2 cells to CDCA (100 μ mol/L); however, a \sim 2-fold induction of promoter activity by CDCA secondary to FXR/RXR α coexpression was observed (Figure 4A). Although the overall degree of bile acid activation was lower in HepG2 than in LMH cells, a comparison of different bile acids with respect to their potency in inducing promoter activity indicated a similar rank order for the hydrophobic bile acids CDCA > DCA > LCA (Figure 4B). Electrophoretic mobility shift assays showed binding of heterodimeric FXR/RXR α to the IR-1 element, not only using *in vitro* translated FXR/RXR α proteins (Figure 5), but also when total nuclear protein from LMH cells or FXR/RXR α -transfected HepG2 cells was used (Figure 6). The effect of different concentrations of CDCA on OATP8 promoter activation was evaluated in both LMH and HepG2 cells. Interestingly, submaximal activation was seen at 10 μ mol/L (HepG2) and 25 μ mol/L (LMH), whereas at concentrations of 200 μ mol/L, activation was no longer evident (Figure 7). These results essentially support the data of Makishima et al.,¹¹ who showed a half-maximal effective concentration of 10 μ mol/L CDCA for human FXR in HepG2 cells. Finally, induction of endogenous OATP8 gene expression by bile acids was shown in cultured Huh7 cells by real-time PCR. CDCA-treated cells had a 71- to 92-fold increase in the amount of OATP8 mRNA compared with DMSO-treated control cells (Table 2), suggesting a physiologic role of the FXR pathway for OATP8 gene regulation.

Genes that have been shown to bind FXR in their promoter region and to be activated by bile acids include the hepatocellular BSEP,¹⁵ the ileal cytosolic bile acid binding protein,²⁵ the phospholipid transfer protein,^{23,26} phenylethanolamine N-methyltransferase,²³ and carnitine palmitoyl-transferase II.²³ The present study adds the human organic anion transporting polypeptide 8 to the list of genes that bind FXR/RXR α and are activated by bile acids. Induction of BSEP gene expression by FXR promotes the efflux of bile acids from hepatocytes in situations in which the flux of bile acids through the hepatocyte is increased (e.g., during biliary diversion or bile acid feeding),^{39,40} or in which intracellular bile acid concentrations increase (e.g., during cholestasis).⁴¹ Coordinately, the major hepatocellular uptake system for bile acids, NTCP, and the key enzyme in the synthesis of bile acids from cholesterol, cholesterol-7 α -hydroxylase (cyto-

chrome P450 enzyme 7A1), are down-regulated by bile acids through a pathway involving FXR-mediated activation of short heterodimer partner 1.^{8,9,16,42} These mechanisms aim to protect the hepatocyte from the potentially toxic effect of elevated intracellular bile acid concentrations.

One can only speculate as to the physiologic significance of OATP8 gene induction by FXR and bile acids. The FXR-mediated changes in the expression of genes involved in bile acid transport (NTCP, BSEP) or synthesis (cytochrome P450 enzyme 7A1) are consistent with the changes in gene expression that occur during cholestatic liver injury.^{43,44} Thus, expression of the basolateral transporters NTCP and OATP-C is decreased in cholestasis.^{45,46} Because OATP8 is a multispecific hepatocellular uptake system for organic anions, xenobiotics, and selected oligopeptides,⁴⁻⁶ the purpose of FXR-mediated induction of OATP8 expression in conditions in which other basolateral uptake systems are inactivated could be to maintain ongoing hepatic uptake of xenobiotics and peptides. This could preserve the liver's metabolic function in xenobiotic disposal even during cholestasis. Increased uptake of (regulatory) peptides during cholestasis could even serve as a protective mechanism. Alternatively, OATP8 could function as a sinusoidal export system in cholestasis. OATPs are thought to be anion exchangers, as evidenced by bidirectional transmembrane bromosulphophthalein transport in rat Oatp1 (Slc21a1) expressing HeLa cells⁴⁷ and by rat Oatp1-mediated taurocholate/HCO₃⁻, and taurocholate/glutathione exchange in HeLa cells and *Xenopus laevis* oocytes, respectively.^{48,49} Teleologically, the induction of a basolateral transport system that is capable of effluxing cholephilic organic anions could represent a protective mechanism that prevents the hepatocellular accumulation of potentially toxic metabolites in cholestasis.

A further example for the induction of a basolateral OATP by a bile acid is the regulation of mouse Oatp2 (Slc21a5) by the PXR ligand LCA. PXR induces Oatp2 expression secondary to LCA treatment, a regulatory pathway shown to be defective in PXR^{-/-} mice.⁵⁰ PXR is proposed as a physiologic sensor of LCA concentrations in the enterohepatic circulation that promotes both uptake of LCA via Oatp2 and detoxification via Cyp3a11. Mouse Oatp4 (Slc21a6), which is the only known mouse orthologue of both human OATP-C (SLC21A6) and OATP8 (SLC21A8), also possesses an IR-1 element at nt -76 to -88⁵¹ with the identical hexameric repeats (AGGACA_gTGACCT) present in the OATP8 promoter. Based on the results of this study, it will be of interest to study expression of Oatp4 in FXR^{-/-} mice fed a diet

supplemented with cholic acid.⁵² Parenthetically, the promoter of the human OATP-C gene does not contain an IR-1 element and the OATP-C promoter constructs described previously⁷ are not transactivated by bile acids (data not shown).

In conclusion, this study provides evidence for the regulation of the human OATP8 gene by FXR/BAR and for its activation by bile acids. OATP8 is the first known basolateral uptake system of hepatocytes identified as a target of FXR/BAR-mediated induction. It could act as a rescue uptake system for xenobiotics, organic anions, and peptides that is regulated divergently to other basolateral transport proteins when intracellular bile acids are elevated.

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