Nuclear receptor-mediated repression of human cholesterol 7α-hydroxylase gene transcription by bile acids

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Abstract **Hydrophobic bile acids strongly repressed transcription of the human cholesterol 7α-hydroxylase gene (*CYP7A1*) in the bile acid biosynthetic pathway in the liver. Farnesoid X receptor (FXR) repressed *CYP7A1*/Luc reporter activity in a transfection assay in human liver-derived HepG2 cells, but not in human embryonic kidney (HEK) 293 cells. FXR-binding activity was required for bile acid repression of *CYP7A1* transcription despite the fact that FXR did not bind to the *CYP7A1* promoter. FXR-induced liver-specific factors must be required for mediating bile acid repression. Bile acids and FXR repressed endogenous CYP7A1 but stimulated α-fetoprotein transcription factor (FTF) and small heterodimer partner (SHP) mRNA expression in HepG2 cells. Feeding of rats with chenodeoxycholic acid repressed CYP7A1, induced FTF, but had no effect on SHP mRNA expression in the liver. FTF strongly repressed *CYP7A1* transcription in a dose-dependent manner, and SHP further inhibited *CYP7A1* in HepG2 cells, but not in HEK 293 cells. FXR only moderately stimulated SHP transcription, whereas FTF strongly inhibited *SHP* transcription in HepG2 cells. Results revealed that FTF was a dominant negative factor that was induced by bile acid-activated FXR to inhibit both *CYP7A1* and *SHP* transcription. Differential regulation of FTF and SHP expression by bile acids may explain the wide variation in CYP7A1 expression and the rate of bile acid synthesis and regulation in different species.**—Chen, W., E. Owsley, Y. Yang, D. Stroup, and J. Y. L. Chiang. **Nuclear receptor-mediated repression of human cholesterol 7α-hydroxylase gene transcription by bile acids.** ***J. Lipid Res.*** **2001.** 42: **1402–1412.**

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Conversion of cholesterol to bile acids occurs exclusively in the liver and is regulated by the rate-limiting enzyme cholesterol 7α-hydroxylase, which is a product of the *CYP7A1* gene (1). The rate of bile acid synthesis and CYP7A1 protein activity are feedback inhibited by bile acids returning to the liver via enterohepatic circulation of bile. Many lines of evidence have suggested that *CYP7A1* transcription is inhibited by hydrophobic bile acids (2–5). We have previously demonstrated that hydrophobic bile acids strongly repress *CYP7A1* transcription, in transient transfection assays in confluent HepG2 cells, and identified a bile acid response element (BARE-II, nucleotides −148 to 118) (6, 7). This sequence contains a direct repeat of an AGGTCA-like motif separated by one base (DR1) that has been identified as a binding site for hepatocyte nuclear factor 4 (HNF4) (8). This HNF4 site overlaps with a binding site (TCAAGGCCA) for the NR5A2 family of monomeric nuclear receptors (9). NR5A2 receptors include rat α-fetoprotein transcription factor (FTF) (9), mouse liver-related homolog (LRH) (9), human cholesterol 7α-hydroxylase promoter factor (CPF, and variants) (10), and human B1-binding factor (hB1F) (11).

Farnesoid X receptor (FXR) is activated by hydrophobic bile acids such as chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) at physiological concentrations (12–14). Bile acid-activated FXR represses *CYP7A1* transcription in transfection assays in HepG2 cells (12, 15). The potency of bile acid repression of the *CYP7A1* gene correlates with the efficacy of bile acid activation of FXR. We have shown that FXR represses rat *CYP7A1* transcription through the BARE-II, but FXR does not bind to this sequence (15). No FXR response element (inverted repeat with one-base spacing, IR1) has been found in *CYP7A1*. We suggest that FXR represses *CYP7A1* transcription by an indirect mechanism involving other bile acid receptors in hepatocytes (15).

Abbreviations: BARE-I and -II, bile acid response elements I and II; CA, cholic acid; CDCA, chenodeoxycholic acid; *CYP7A1*, cholesterol 7α-hydroxylase gene; *CYP8B1*, sterol 12α-hydroxylase gene; DCA, deoxycholic acid; DR, direct repeat; EMSA, electrophoretic mobility shift assay; FTF, α-fetoprotein transcription factor; FXR, farnesoid X receptor; HEK, human embryonic kidney; HNF4, hepatocyte nuclear factor 4; IBABP, ileum bile acid-binding protein; LXR, liver orphan receptor; Luc, luciferase; NTCP, Na^+-taurocholate cotransport protein; RXR, retinoid X receptor; SHP, small heterodimer partner; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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While this manuscript was in preparation, two laboratories reported that bile acids and FXR antagonist repressed CYP7A1 but stimulated small heterodimer partner (SHP) mRNA expression in mouse liver (16, 17). SHP is predominantly a negative nuclear receptor that interacts with FTF and inhibits *CYP7A1* transcription. This is consistent with impaired bile acid synthesis and increased SHP mRNA expression in the *fxr* knockout mouse model (18). The objective of this research was to identify liver-specific factors that are induced by bile acids and mediate bile acid inhibition of regulatory genes in bile acid synthesis. We studied FXR-mediated bile acid repression of the human *CYP7A1* gene and revealed a complex regulatory repertoire involving FXR induction of FTF, which functions as a negative factor that interacts with and regulates *SHP* and *CYP7A1* transcription.

EXPERIMENTAL PROCEDURES

Materials

Human hepatocellular blastoma cells HepG2 (ATCC HB8065), human embryonic kidney (HEK) 293 cells (ATCC CRL1573), intestinal Caco-2, and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD). DMEM-F12 and trypsin-EDTA were purchased from Life Technologies (Rockville, MD). Penicillin G-streptomycin and fetal calf serum were from Celox (Hopkins, MN) and Irvine Scientific (Santa Ana, CA), respectively. Bile acids and their taurine conjugates were supplied by Sigma (St. Louis, MO). Restriction enzymes and other modifying enzymes were purchased from BRL (Bethesda, MD) or Promega (Madison, WI). Luciferase (Luc) reporter gene vectors pGL3 and the reagents for luciferase assays were purchased from Promega. The DNA purification kit was from Clontech (Palo Alto, CA). The double-stranded oligonucleotides were synthesized by Life Technologies. The RETROscript kit for RT-PCR was purchased from Ambion (Austin, TX). TRI reagent for RNA isolation was purchased from Sigma.

Methods

Reporter gene and receptor expression plasmids. Human *CYP7A1*/Luc reporter gene, ph-1887/Luc and ph-372/Luc, and rat p-376/Luc were constructed as described previously (19, 20). An *SHP*/Luc reporter plasmid containing 2,080 bp of 5′ upstream sequence of human *SHP* and a mouse *IBABP*/Luc reporter plasmid containing nucleotides −496 to +40 of the gene encoding mouse ileum bile acid-binding protein (IBABP) were kindly provided by D. Moore (Baylor College of Medicine, Houston, TX). A mutant FXR (mFXR) lacking DNA-binding activity was constructed by changing Cys-179 of the Zn^{2+} finger of rat FXR to an arginine. This was based on the finding that mutation of the corresponding cysteine residue in HNF4 generated a dominant negative HNF4 mutant lacking DNA-binding activity (21). This cysteine-to-arginine conversion was accomplished by introducing a T→C point mutation in the rat FXR sequence by PCR with the 3′ mutagenic primer (GCACTTCCTTAGCCGGC**G**ATCCTG, mutation in boldface, *Nae*I restriction site underlined), and the T7 sequencing primer (5′ primer) (Promega), using pcDNA3FXR as a template. The resulting fragment was digested with *Kpn*I and *Nae*I and was assembled into pcDNA3 cut with *Kpn*I and *Bam*HI, with the remainder of the FXR-coding region on an *Nae*I-*Bam*HI fragment. The clones were screened for the mutation by detecting the created *Mbo*I restriction site by restriction digestion (aggat**t**gccggc to aggat**c**gccggc; mutation in boldface, *Nae*I restriction site underlined).

Expression plasmid for human retinoid X receptor α (pCMX-hRXRα), rat FXR (pRSVFXR), human FXR (pcDNAhFXR), human liver Na^+-taurocholate cotransporter protein (NTCP), mouse SHP (CDM8mSHP) and human FTF (CDM8FTF), and human CPF (pcDNA3CPF) were kindly provided by R. Evans (Salk Institute, La Jolla, CA), C. Weinberger (National Institute of Environmental Health Sciences, Research Triangle Park, NC), D. Mangelsdorf (UT Southwestern Medical School, Dallas, TX), P. Dawson (Wake Forest University, Winston-Salem, NC), D. Moore, and B. Shan (Tularik, South San Francisco, CA), respectively. Rat FXR was moved from pRSVFXR to pCDNA3 vector to generate pCDNA3-rFXR.

Cell culture and transient transfection assay. HepG2 cells were cultured in DMEM-F12 (50:50) supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Cells were grown in 12-well plates to confluence in 3 to 4 days. Cells were grown in serum-free medium after transient transfection of DNA by the calcium phosphate DNA coprecipitation method. The amount of plasmid used was 2.5 μg of reporter and 0.5 μg of receptor expression plasmid in all assays, except in the dose-response study, in which various ratios of reporter to expression vector were used. In each assay, 0.5 μg of pCMVβ-galactosidase was cotransfected as an internal standard for normalization of transfection efficiency. Four hours after transfection, cells were treated with bile acids or vehicle (ethanol). Cells were harvested 40 h later, washed twice with phosphate-buffered saline, and lysed with reporter lysis buffer (Promega). Luciferase activities were measured by luminometer and normalized by dividing the relative light units (RLU) by β-galactosidase activity. Each assay was done in triplicate and each experiment was repeated at least three times. HEK 293 and CHO cells were transfected in the same method, except that CHO cells were plated 1 day before transfection. Statistic analyses were performed between treated and untreated control, using Student's *t*-test.

Determination of mRNA by RT-PCR. HepG2, HEK 293, and CHO cells were grown in DMEM-F12 (50:50) supplemented with 10% (v/v) heat-inactivated fetal calf serum and penicillin G (100 U/ml) and streptomycin (100 μg/ml). Cells were grown in 25-cm^2 flasks to confluence in 3 to 4 days. FXR and RXRα expression plasmids (10 μg each) were transiently transfected by the calcium phosphate-DNA coprecipitation method. Empty plasmid, pcDNA3 for CPF, FXR, and RXR, or CDM8 for FTF and SHP, was transfected as a control, or added to compensate for the total amount of DNA used in each transfection assay. Cells were then treated with 25 μM cholic acid (CA) or CDCA 4 h after transfection and were harvested 40 h after treatment. Total RNAs were isolation with TRI reagent. A RETROscript kit (Ambion) was used to quantify, by RT-PCR, CYP7A1 mRNA levels in HepG2, HEK 293, and CHO cells. Total RNAs were primed with decamer provided in the kit and cDNA was synthesized by reverse transcriptase, followed by PCR with gene-specific primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Rig/S15 mRNAs were amplified as internal standards of PCR. Primer sets were designed to amplify a cDNA fragment containing sequences derived from two adjacent exons to distinguish them from potential PCR products due to contamination of genomic DNA in RNA preparations. Human FXR-specific primers were as follows: forward primer, 5′-CAGAATTCACAGTGAAGGTCGT GACTTGC-3′; reverse primer, 5′-GCGGTACCGTGGTGATGAT TGAATGTCC-3′. Human CYP7A1-specific primers were as follows: forward primer, 5′-GCATCATAGCTCTTTACCCAC-3′; reverse primer, 5′-GGTGTTCTGCAGTCCTGTAAT-3′ (22). GAPDH primers were as follows: forward primer, 5′-CCATCACCATCTTC CAGGAG-3′; reverse primer, 5′-GGATGATGTTCTGAGAGCC-3′. The sizes of product amplified with the FXR, CYP7A1, GAPDH, and Rig/S15 primers (provided in the RETROscript

kit) were 672, 416, 415, and 361 bp, respectively. These mRNAs could be detected by 20 cycles of PCR and were linear up to 30 cycles of PCR.

Determination of mRNA by Northern blot hybridization. Female Sprague-Dawley CD rats were fed a diet supplemented with 1% CDCA for 2 weeks. Rats were housed in a room with a reversed light cycle (3 AM to 3 PM dark, and 3 PM to 3 AM light) and killed at 9 AM. Total and poly(A^+) RNAs were isolated from livers, using an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) and a poly(A^+) Pure mRNA isolation kit (Ambion). HepG2 cells were grown to confluence in a 75-cm^2 flask. Cells were transfected with 10 μg each of pcDNAhFXR and pcCMVhRXR by Tfx-20 reagent (ratio 1:3, w/v; Promega). Cells were treated with 25 μM CDCA 1 h after transfection and collected 40 h later for isolation of total RNA, using the Ultraspec-II RNA isolation system according to the manufacturer instructions. For Northern blot, 20 μg of total RNA was separated on a 1.2% formaldehyde agarose gel, transferred to Nylon plus filter (MSI, Westboro, MA), and hybridized with CYP7A1, mouse SHP, or human FTF cDNA labeled by the Klenow fragment of DNA polymerase I with [^{32}P]dCTP. The same membrane was reused for hybridization with other probes after stripping out of radioactivity. Radioactivity was detected by PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic mobility shift assay (EMSA). Oligonucleotides were custom synthesized (upper strand indicated): human SHP (gatc GAGTTAaTGACCTgatc), mouse SHP (gatcGGGTTAaTGAC CCgatc), and FTF-binding sites (underlined) of rat CYP7A1 (gatc GTTCAAGGCCAGTTACTACCAgatc) and human CYP7A1 (gatGT TCAAGGCCGGGTAATGCTAgatc). Double-stranded synthetic probes for EMSA were prepared by heating equal molar amounts of complementary synthetic oligomers to 100°C in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), holding at 88°C for 10 min, and then allowing the hybridization mix to cool to ambient temperature in a heating block. The resulting double-stranded fragments were end labeled by incorporating α[^{32}P]dCTP (3,000 Ci/mol) with the Klenow fragment of DNA polymerase I. Oligonucleotides blunted with nonlabeled dNTPs were used as unlabeled competitors in EMSA. Labeled fragments were isolated from a 15% polyacrylamide gel. DNA-binding reactions were initiated by the addition of 100,000 cpm of the probe to protein. Reaction mixtures (20 μl) were preincubated with 1 μg of poly(dI-dC)·poly(dI-dC) and 40 pmol of single-stranded synthetic oligomer dissolved in 1× gel shift buffer (containing 12 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 15% glycerol) in a 20 μl reaction mixture, and incubated for 30 min at 25°C. FXR, RXRα, SHP, and CPF were synthesized in vitro with a Quick Coupled Transcription/Translation system (Promega) according to the manufacturer instructions. Samples were run on 4% polyacrylamide gels, dried, autoradiographed by PhosphorImaging, and quantified with IP Lab Gel software (Signal Analytics, Vienna, VA).

RESULTS

Effect of bile acids, liver bile acid transporter, and FXR/RXR on human *CYP7A1*/Luc reporter activity in HepG2 cells

We reported previously that physiological concentrations of bile acids were able to inhibit rat *CYP7A1* transcription in HepG2 cells and that cotransfection with FXR enhanced bile acid repression (15). Here we have carried out a detailed study of bile acid inhibition of human *CYP7A1*/Luc reporter activity. As shown in **Fig. 1A**, physiological (25 μM)

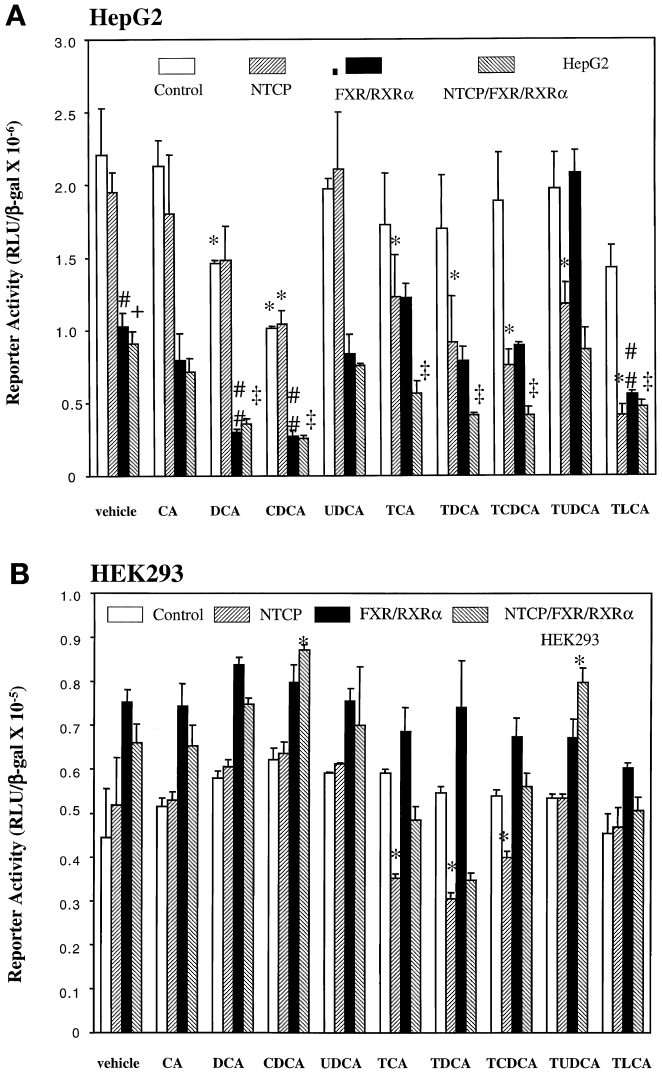

Fig. 1. Effects of bile acids, liver bile acid transporter, and FXR/RXRα on human *CYP7A1* transcription. A: Transfection assays in HepG2 Cells. HepG2 cells were cultured to confluence. Bile acids (CA, DCA, CDCA, and UDCA) and their taurine conjugates (TCA, TDCA, TCDCA, TUDCA, and TLCA) (25 μM) were added individually to HepG2 cells transfected with human *CYP7A1* (−1887)/Luc plasmid containing 1,887 bp of 5′ upstream sequence, with or without cotransfection with NTCP, FXR/RXRα, or FXR/RXRα/NTCP and treated with the indicated bile acids. Cultures were harvested 40 h later for assay of luciferase activity as described in Experimental Procedures. Ethanol was used as a vehicle for delivery of bile acids. Error bars indicate standard errors of the means of triplicate assays. Graph shown is a representation of three separate experiments. CA, Cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; TCA, tauro-CA; TDCA, tauro-DCA; TCDCA, tauro-CDCA; TUDCA, tauro-UDC; TLCA, taurolithocholic acid. B: Transfection assays in HEK 293 cells. HEK 293 cells were cultured to confluence, transfected with the *CYP7A1* (−1887)/Luc construct with or without cotransfection with NTCP, FXR/RXRα, or FXR/RXRα/NTCP, and treated with the indicated bile acids (25 μM). Experiments and analysis were the same as in (A). The asterisk (*) indicates a significant difference ($P < 0.05$) from vehicle (ethanol) by bile acid treatment. The symbols $^{\#}$ and $^{+}$ indicate a significant difference ($P < 0.05$) from control (open columns) by transfection with FXR/RXRα and NTCP/FXR/RXRα, respectively. The symbols $^{\#\#}$ and ‡ indicate a significant difference ($P < 0.05$) from vehicle by bile acid treatment when transfected with FXR/RXRα and NTCP/FXR/RXRα, respectively.

concentrations of DCA and CDCA significantly suppressed the human *CYP7A1*/Luc reporter activity by 30% and 50%, respectively, in HepG2 cells. Nonphysiological concentrations up to 75 μM completely inhibited reporter activity (data not shown). Less hydrophobic bile acid, CA, and hydrophilic bile acid, ursodeoxycholic acid (UDCA), had no significant effect on reporter activity. All taurine-conjugated bile acids tested, including taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), and taurolithocholic acid (TLCA), had no effect. When liver bile acid transporter NTCP was cotransfected in HepG2 cells, all taurine-conjugated bile acids significantly repressed reporter activity. Cotransfection of NTCP did not significantly affect the inhibitory action of DCA and CDCA. Apparently taurine-conjugated bile acids require bile acid

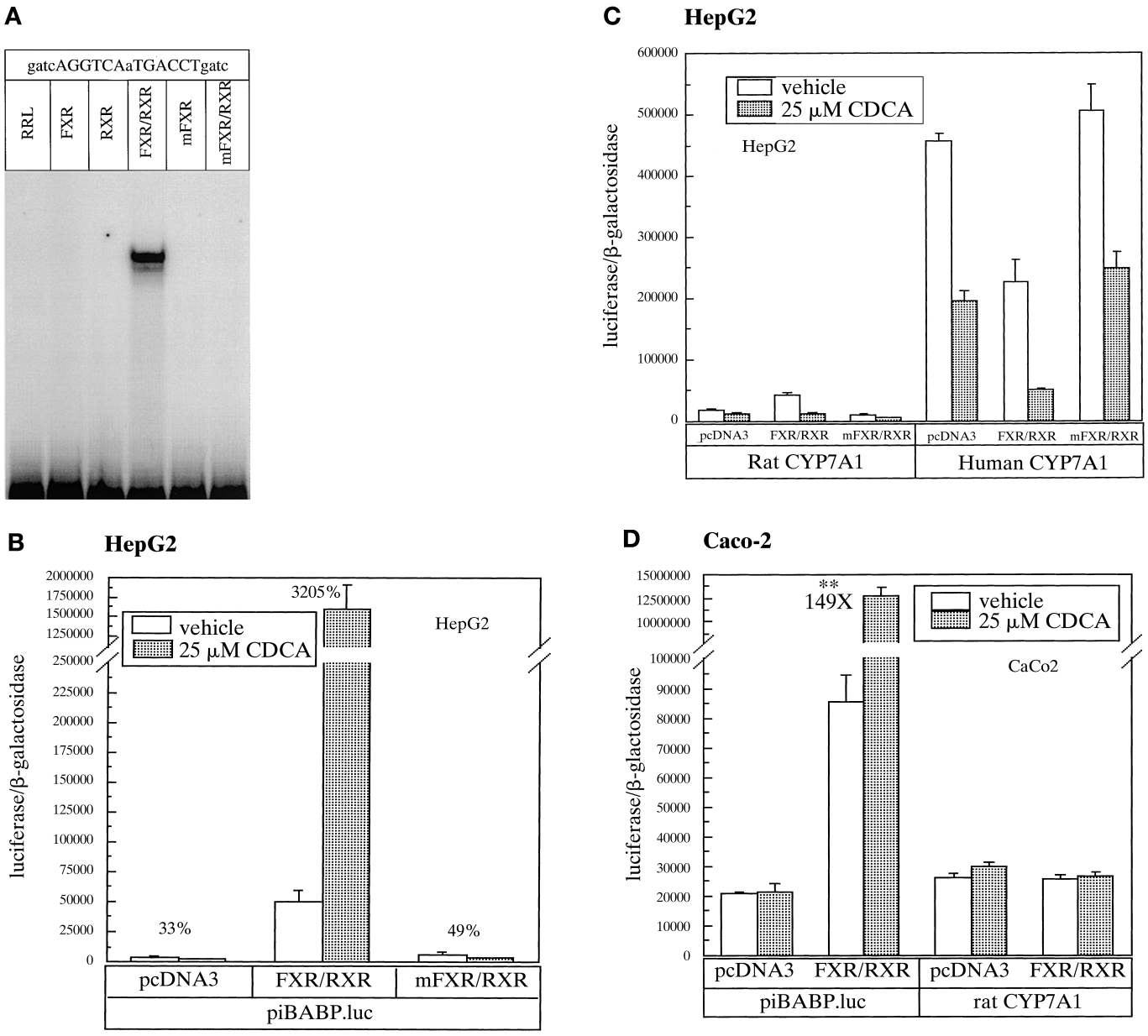

Fig. 2. Effects of CDCA, FXR, and mFXR lacking DNA-binding activity on *IBABP*/Luc and *CYP7A1*/Luc reporter activity. A: Electrophoretic mobility shift assay of FXR, mFXR, and/or RXRα probed with a perfect IR1 oligonucleotide labeled with ^{32}P. RRL, rabbit reticulocyte lysate. B: Human *IBABP*/Luc reporter plasmid was transfected into HepG2 cells. FXR/RXRα expression plasmids (0.5 μg each), mFXR, or control plasmid pcDNA3 was cotransfected as indicated. CDCA (25 μM) or vehicle (ethanol) was added as indicated. The percentages indicate the effect of CDCA, relative to vehicle, in the same group. All groups showed a significant difference with CDCA ($P < 0.05$). C: Rat *CYP7A1* (p-376/Luc) or human *CYP7A1* (ph-372/Luc) reporter plasmid was cotransfected into HepG2 cells with pcDNA3, FXR/RXRα, or mFXR/RXRα as indicated. CDCA (25 μM) or vehicle (ethanol) was added as indicated. CDCA significantly reduced reporter activity in each group ($P < 0.05$). D: Caco-2 cells were used for the transfection assay. The *IBABP*/Luc or rat p-376/Luc reporter plasmid was cotransfected with FXR/RXRα expression plasmids. The pcDNA3 plasmid was used as a control. CDCA (25 μM) or vehicle (ethanol) was added as indicated. X, Fold change by CDCA. Other groups are not altered significantly by CDCA treatment. Experimental procedures and analysis of data are the same as in Fig. 1. ** Statistically significant difference ($P < 0.005$) compared with vehicle.

transporter for transport, whereas hydrophobic bile acids were able to diffuse into HepG2 cells (7). When cotransfected with the FXR/RXRα expression vectors (5:1 ratio of reporter to receptor plasmid), *CYP7A1* reporter activity was repressed 50%. This is in contrast to the stimulatory effect of FXR on rat *CYP7A1* (15). Addition of 25 μM DCA, CDCA, TDCA, TCDCA, or TLCA further repressed human *CYP7A1*/Luc reporter activity up to 80%. When cotransfected with both FXR/RXRα and NTCP, all bile acids except UDCA and TUDCA further suppressed *CYP7A1*/Luc reporter activity. Therefore, hydrophobic bile acids strongly repress human *CYP7A1* transcription, and overexpression of FXR further represses reporter activity in HepG2 cells. This indicates that bile acid repression of *CYP7A1* is mediated either by endogenous FXR or by other factors activated by bile acid in liver cells.

To test whether the bile acid effect on *CYP7A1* transcription is liver specific, we performed the same experiments in HEK 293 cells (Fig. 1B). In contrast to the results obtained in HepG2 cells, these bile acids did not inhibit reporter activity, with or without cotransfection with FXR and/or NTCP. This is consistent with our previous observation that bile acid inhibition of *CYP7A1* transcription is liver specific (20). It appears that liver-specific factors other than FXR were required for bile acid inhibition.

DNA-binding activity of FXR was required for FXR-mediated regulation of gene transcription

As a positive control, effects of FXR and CDCA on *IBABP* transcription were studied in HepG2 cells and also in intestinal Caco-2 cells, using the same transfection system for studying *CYP7A1* transcription. An mFXR, with a cysteine residue of the Zn^{2+} finger converted to an arginine, was obtained by mutagenesis. This mFXR was not able to bind to an IR1 probe, whereas the wild-type FXR forms a heterodimer with RXRα and bound strongly to the probe (**Fig. 2A**). Figure 2B shows that cotransfection with FXR strongly stimulated human *IBABP*/Luc reporter activity by about 10-fold in HepG2 cells. Addition of 25 μM CDCA further stimulated *IBABP* reporter activity to 32-fold. The mFXR had no effect on *IBABP* reporter activity, indicating that DNA-binding activity of FXR was required for the transactivation of *IBABP*. DNA-binding activity of FXR was also required for the enhancement of the bile acid repression of both rat and human *CYP7A1*/Luc reporter activity in HepG2 cells (Fig. 2C). Wild-type FXR stimulated rat but inhibited human *CYP7A1*, as we observed previously (15). Addition of CDCA resulted in enhanced repression of both rat and human *CYP7A1*. On the other hand, the mFXR had no effect on rat or human *CYP7A1*/Luc reporter activity. These results could be explained by FXR being a positive transcription factor that stimulates rat *CYP7A1* via binding to a DR4 in the BARE-I, as reported previously (15). Activation of FXR by bile acids may upregulate a negative factor or downregulate a positive factor, thus indirectly repressing *CYP7A1* transcription in hepatocytes. Human *CYP7A1*, which lacks the DR4, was repressed by FXR and bile acids, consistent with the indirect mechanism we have proposed (15). In intestinal Caco-2 cells (Fig. 2D), wild-type FXR stimulated *IBABP* reporter activity by 4-fold, and

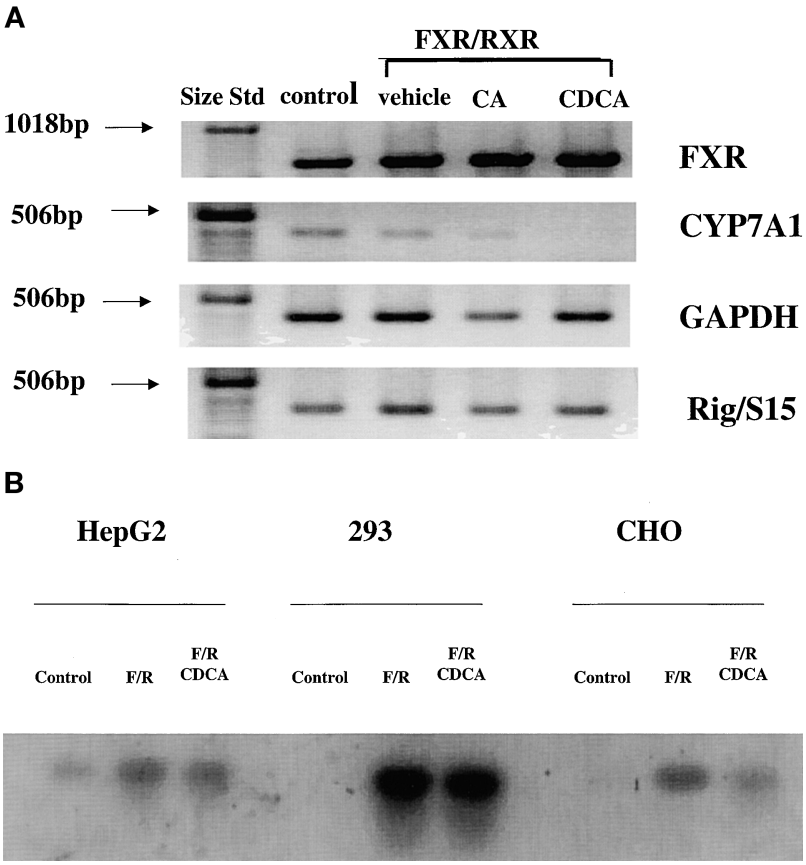

Fig. 3. RT-PCR analysis of the effect of FXR and bile acids on *CYP7A1* and mRNA expression levels in HepG2 cells. A: HepG2 cells were cultured to confluence, transfected with FXR/RXRα, and treated with 25 μM CA or CDCA or vehicle (ethanol). Control was transfected with pcDNA3 empty plasmid. Forty hours later, the cells were harvested and used for isolation of RNA. RT-PCR was performed as described in Experimental Procedures. B: FXR mRNA expression in HepG2, HEK 293, and CHO cells. HepG2, HEK 293, and CHO cells were cultured to confluence, transfected with or without FXR/RXRα (F/R), and treated with 25 μM CDCA. Forty hours later, the cells were harvested and used for RNA isolation. RT-PCR (25 cycles) was performed as described in Experimental Procedures.

addition of CDCA dramatically stimulated *IBABP* reporter activity by 149-fold. In contrast, rat *CYP7A1*/Luc reporter was not regulated by CDCA and FXR in Caco-2 cells, consistent with a mechanism in which bile acid repression requires the induction of liver-specific factors by bile acids.

Effect of bile acids and FXR/RXRα on CYP7A1 and FXR mRNA expression levels

To study the effect of FXR and bile acids on human CYP7A1 mRNA expression, we transfected HepG2 cells with FXR expression plasmid and measured CYP7A1 mRNA expression levels. Endogenous FXR and CYP7A1 mRNA levels in HepG2 cells were too low to be detected by hybridization. Therefore, RT-PCR was used. As shown in **Fig. 3A**, endogenous FXR mRNA in HepG2 cells could be detected by 25 cycles of amplification. Transfection with FXR/RXRα increased FXR mRNA expression in HepG2 cells by about 3-fold. Transfection with FXR/RXRα suppressed CYP7A1 mRNA expression by about 50%. Addition of 25 μM CA further reduced CYP7A1 mRNA by 80%. CDCA (25 μM) completely inhibited CYP7A1 mRNA expression. FXR mRNA was not detected in HEK 293 and CHO cells (Fig. 3B) and was highly expressed by transfection with FXR/RXRα because of the high transfection efficiency of these two cell lines. These results demonstrated that bile acids and FXR strongly repressed CYP7A1 mRNA expression in HepG2 cells.

Effect of bile acids and FXR on FTF and SHP mRNA expression in HepG2 cells and rat livers

Because FTF and SHP have been implicated in the regulation of *CYP7A1* transcription, effects of FXR and bile acids on FTF and SHP mRNA expression in HepG2 cells were studied. **Figure 4A** shows FTF and SHP mRNA expression in HepG2 cells by mRNA hybridization. The endogenous FTF and SHP mRNA levels in HepG2 cells were low. When CDCA (25 μM) was added, two FTF mRNA transcripts were induced by about 2-fold, but SHP mRNA was not increased. When HepG2 cells were transfected with FXR/RXRα, FTF mRNA levels were increased 8.5-fold in comparison with the untreated control, and SHP mRNA was increased by 2.3-fold.

We also studied the effect of bile acid feeding on FTF and SHP mRNA expression in rat livers (Fig. 4B). As a positive control, feeding of rats with 1% CDCA for 2 weeks repressed CYP7A1 mRNA expression by 70%. To detect FTF or SHP mRNA, poly(A$^+$) RNA was used for hybridization. Feeding of CDCA strongly stimulated FTF mRNA expression by 7-fold. Three FTF transcripts were detected in rat livers. However, CDCA did not affect SHP mRNA expression in rat livers. This is in contrast to the strong induction of SHP mRNA expression by 7-fold, but no effect on LRH-1 mRNA, in mouse livers (16).

Effects of FTF and SHP on *CYP7A1*/Luc reporter activity

Because FTF and SHP are induced by bile acids and FXR, these two nuclear receptors might directly inhibit *CYP7A1* transcription. Therefore, the effects of FTF and SHP on human *CYP7A1*/Luc reporter activities in HepG2 cells were studied. In our transfection assay, a reporter plasmid (2.5 μg)-to-receptor plasmid (0.5 μg) ratio of 5 to 1 was routinely used to avoid possible artifacts caused by a large excess of receptor expressed. Transfection with FTF repressed human *CYP7A1*/Luc reporter activity by 50%,

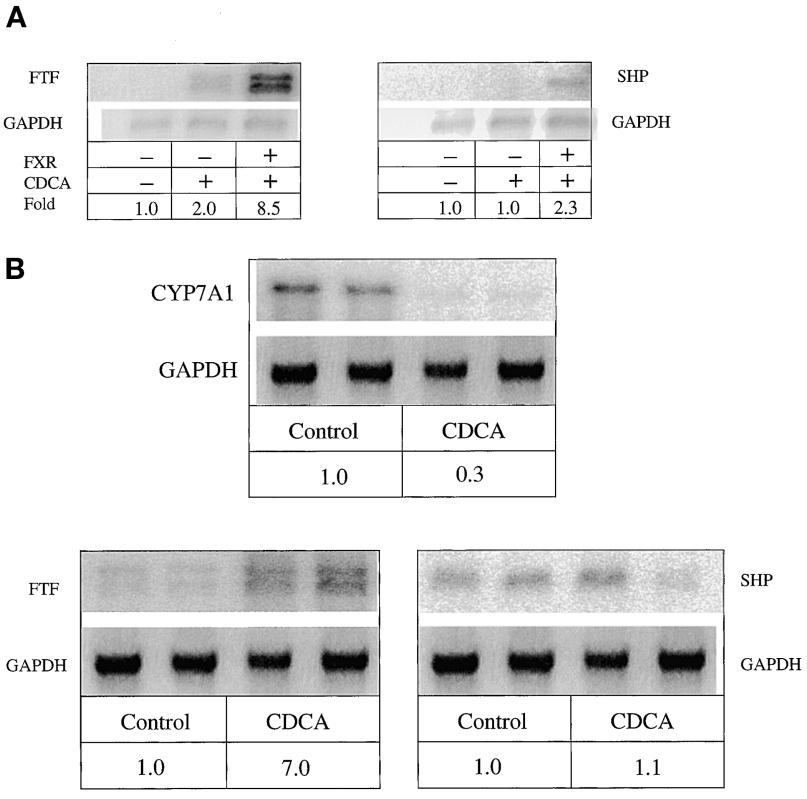

Fig. 4. Effects of FXR and CDCA on the expression of FTF and SHP mRNA in HepG2 cells (A) and rat livers (B). A: HepG2 cells were grown in 75-cm^2 flasks to confluence and transfected with FXR/RXRα expression plasmid (10 μg each). CDCA (25 μM) was added as indicated. Cells were harvested 40 h later. Total RNAs were isolated from cells and separated on a 1% formaldehyde-denatured agarose gel for Northern hybridization. The ^{32}P-labeled *FTF* or *SHP* cDNA probe was used to hybridize the membrane separately. The same membrane was used for hybridization after stripping out of radioactivity. The sizes of FTF and SHP mRNA are about 65 and 25 kb, respectively. B: Total RNA (for CYP7A1) and poly(A$^+$) RNAs (for FTF and SHP) were isolated from the livers of rats fed with 1% CDCA and untreated control. Twenty micrograms of total RNA and 5 μg of poly(A$^+$) RNA were used in Northern blots. Fold change was estimated by scanning each band against the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and averaged.

but CPF had less effect on reporter activity (**Fig. 5A**). FTF (541 amino acid residues) and CPF (495 amino acid residues) may have different activity due to differences in the N-terminal sequences. When SHP and CPF or FTF were cotransfected, reporter activity was repressed by about 70% to 80%.

When the same assays were done in HEK 293 cells (Fig. 5B), FTF slightly stimulated reporter activity. CPF significantly stimulated reporter activity by 2-fold, similar to that observed by Nitta et al. (10). Interestingly, cotransfection of SHP with FTF or CPF did not repress reporter activity. Again, this illustrates that regulation of *CYP7A1* transcription by bile acids and nuclear receptors is a liver-specific event.

Figure 5C shows that FTF strongly repressed *CYP7A1* reporter activity in a dose-dependent manner, and addition of SHP synergistically repressed the activity in HepG2

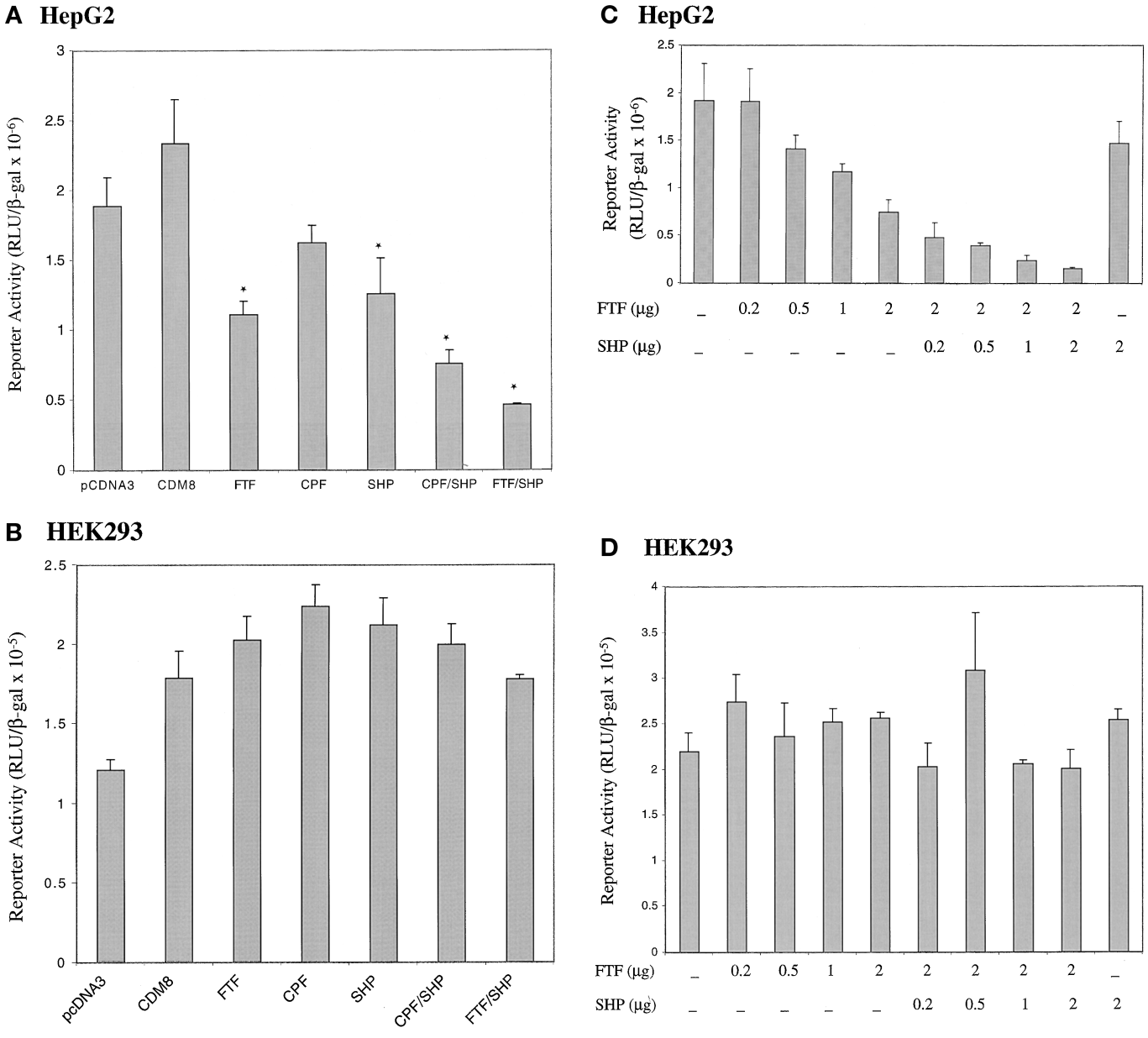

Fig. 5. Effects of FTF, CPF, and SHP on human *CYP7A1*/Luc reporter activity. A: HepG2 cells were grown to confluence, transfected with a human ph-372/Luc reporter plasmid (2.5 μg), and cotransfected with *FTF*, *CPF*, and/or *SHP* expression plasmid (0.5 μg each). Cells were harvested 40 h later for assay of luciferase activity. The asterisk (*) indicates a significant difference from control. Empty vector pcDNA3 was used as a control for CPF, and CDM8 was used as a control for FTF and SHP. B: The same assays as in (A) were performed in HEK 293 cells. C: Effects of different amounts of FTF and SHP on human *CYP7A1*/Luc reporter activity. Asterisks (*) indicate a significant difference from control. Amounts of *FTF* or *SHP* expression plasmid used are indicted in cotransfection with *CYP7A1*/Luc plasmid (2.5 μg). Empty vector CDM8 was added to compensate for the amount of expression plasmid added. All data except transfection with 0.2 μg of FTF are significantly different from the control without FTF and SHP but with empty vectors CDM8 and pcDNA3. D: The same assays as in (C) were performed in HEK 293 cells. FTF and/or SHP have no significant effect on reporter activity. Experimental procedure and analysis of data are the same as in Fig. 1.

cells. This dose-dependent inhibitory effect was not observed when assays were done in 293 cells (Fig. 5D). Thus FTF had a dominant negative effect on human *CYP7A1* transcription in HepG2 cells.

EMSA of FXR binding to *SHP* and of CPF binding to *CYP7A1*

Because FXR and CDCA stimulated SHP mRNA expression in HepG2 cells, FXR may bind to the *SHP* gene and activate *SHP* transcription. We analyzed the 5′ upstream sequence of the *SHP* gene and identified a putative IR1 sequence in both mouse *SHP* ($_{-329}$GGGTTAaTGACCC$_{-317}$) and human *SHP* ($_{-291}$GAGTTAaTGACCT$_{-279}$) promoters. To test whether these IR1 sequences bind FXR, we performed an EMSA. As shown in **Fig. 6A**, in vitro-synthesized FXR/RXRα heterodimer bound to the putative IR1 sequence of human *SHP* relatively weakly as compared with a positive control, an IR1 sequence of a well-characterized gene encoding ecdysone heat shock protein 27. This result is consistent with that reported by Goodwin et al. (17). The weak binding of FXR to *SHP* is consistent with the weak stimulatory effect of CDCA and FXR on SHP mRNA expression in HepG2 cells and no effect in rat livers shown in Fig. 4.

An EMSA using in vitro-synthesized CPF was also probed with putative FTF-binding sequences in rat and human *CYP7A1*. The FTF-binding site, $_{136}$TCAAGGCCA$_{-128}$ (human), is located in the BARE-II region. There is a single base substitution of G for A at the 3′ end of the corresponding rat FTF site. As shown in Fig. 6B, both human and rat FTF-binding sites strongly bound in vitro-synthesized CPF.

Effect of FXR on human *SHP*/Luc reporter activity

Because bile acids and FXR stimulate SHP mRNA expression in HepG2 cells and FTF regulates *SHP* transcription, we studied the effect of bile acid, FXR, FTF, and SHP on human *SHP*/Luc reporter activity. **Figure 7A** shows that FXR/RXRα significantly stimulated *SHP*/Luc reporter activity by 30%, whereas FTF repressed *SHP*/Luc reporter activity by 50% in HepG2 cells. FTF had a dominant inhibitory effect over FXR/RXRα. SHP alone did not have any effect on *SHP* reporter activity, but cotransfection with both SHP and FTF strongly repressed *SHP* reporter activity by 90% (Fig. 7A). Thus, the effect of FTF and SHP regulation of SHP transcription is similar to their effects on *CYP7A1*. CDCA (25 μM) significantly repressed *SHP*/Luc reporter activity when FTF was cotransfected.

The same experiments were carried out with HEK 293 cells (Fig. 7B). FTF strongly stimulated *SHP*/Luc reporter activity by 5-fold, in contrast to its inhibitory effect in HepG2 cells. SHP alone had no effect, whereas FXR/RXRα stimulated *SHP* reporter activity by 30% and CDCA enhanced the stimulatory effect. SHP strongly repressed *SHP* reporter activity when cotransfected with FTF. CDCA (25 μM) stimulated *SHP* reporter activity by 2-fold when cotransfected with FXR. Thus FTF is a repressor of *SHP* transcription as assayed in HepG2 cells but an activator in 293 cells.

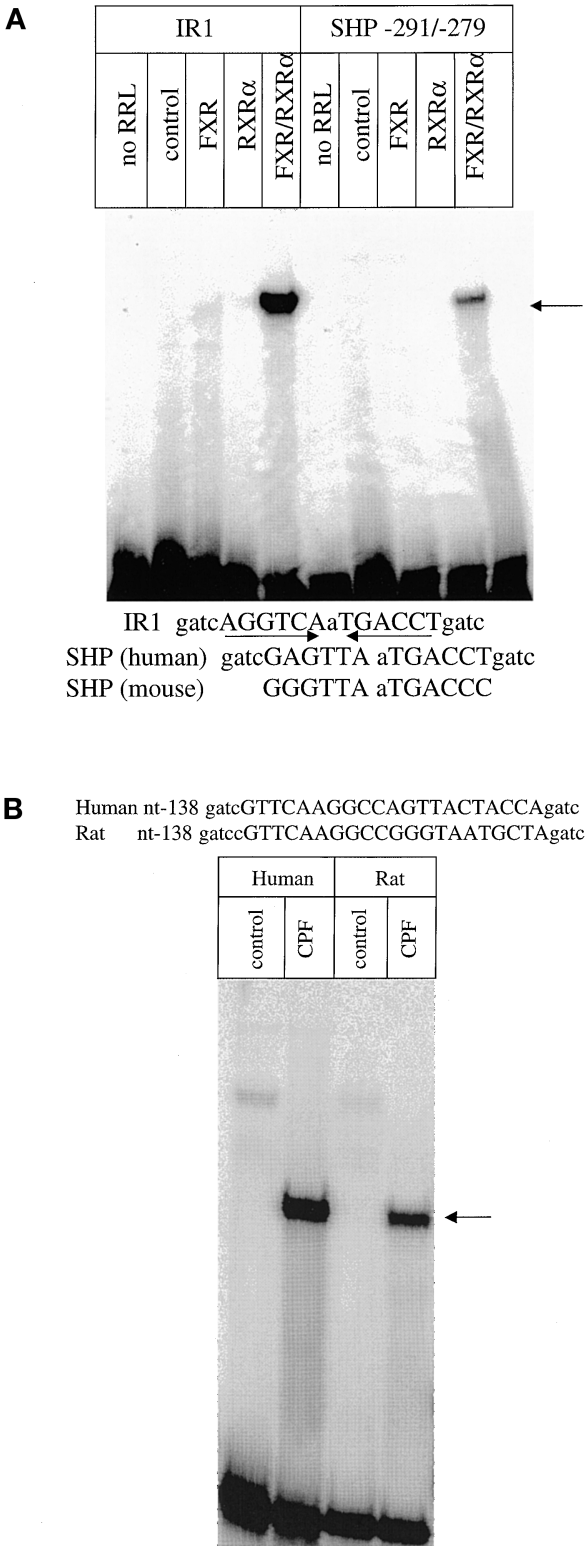

Fig. 6. Electrophoretic mobility shift assays of FXR/RXRα and CPF. A: EMSA of FXR/RXRα probed with a putative IR1 probe in SHP. In vitro-synthesized FXR and/or RXRα were incubated with the ^{32}P-labeled oligonucleotide based on the putative IR1 sequence (SHP −291/−279) of human *SHP*. An IR1 probe of the ecdysone heat shock protein 27 gene was used as a positive control. B: EMSA of CPF probed with putative FTF-binding sites in rat and human *CYP7A1*. In vitro-synthesized CPF was incubated with the ^{32}P-labeled oligonucleotides based on the putative FTF-binding sites in rat and human *CYP7A1*. RRL, rabbit reticulocyte lysate.

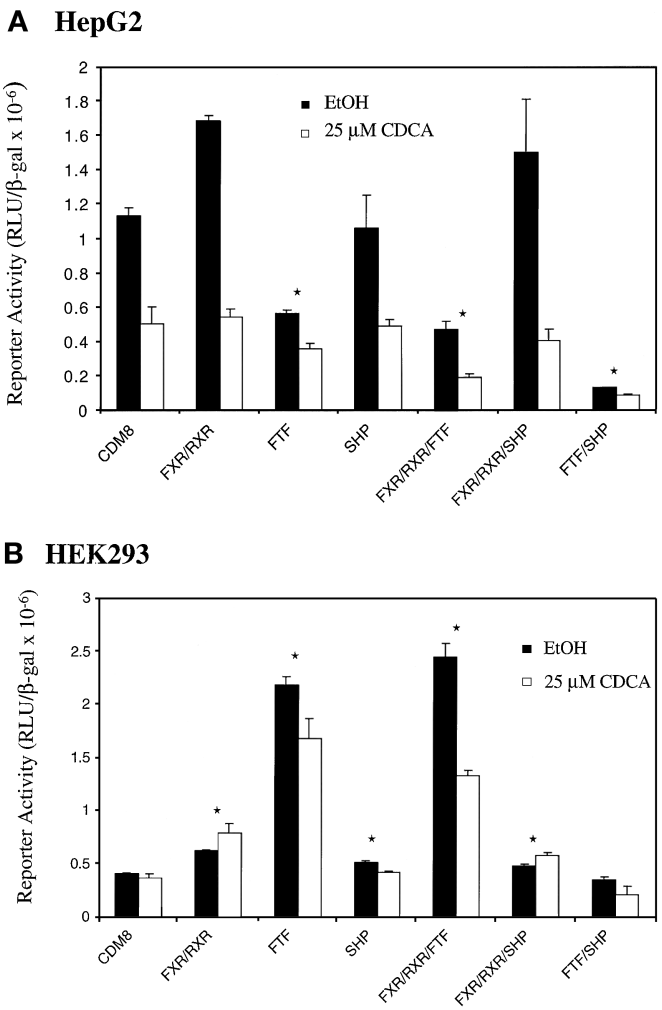

Fig. 7. Effects of SHP, FTF, and FXR/RXRα on *SHP*/Luc reporter activity. A: A human *SHP* (−2080)/Luc reporter plasmid (2.5 μg) containing 2080 bp of SHP 5′ upstream sequence was transfected into HepG2 cells. SHP, FTF, or FXR/RXRα expression plasmids (0.5 μg each) were cotransfected as indicated. Transfection with receptor plasmid significantly affected reporter activities ($P < 0.05$), except SHP alone. B: 293 cells were used for transfection assays as in (A). Transfection with reporter plasmid significantly affected reporter activities ($P < 0.05$), except all assays transfected with SHP. Experimental procedures and analysis of data are the same as in Fig. 1.

DISCUSSION

It is now well recognized that FXR is a highly specific bile acid receptor that is activated by hydrophobic bile acids at physiological concentrations to regulate the genes involved in bile acid synthesis and transport. The positive effect of FXR on its target genes is due to binding of FXR to its unique response elements in these genes. FXR perhaps is the most specific orphan nuclear receptor identified so far. However, the mechanism of negative regulation of *CYP7A1* by FXR is less understood. SHP is a nonspecific negative factor that interacts with many nuclear receptors by either competing for coactivators and/or directly repressing their transactivating activity (23). It appears that SHP interacts with FTF and represses *CYP7A1* transcription. However, our study revealed, by both an in vivo study in rats and an in vitro study in HepG2 cells, that bile acids also stimulate FTF expression. Interestingly, FTF can serve as a negative factor that may directly repress both *CYP7A1* and *SHP* transcription in the liver. Multiple NR5A2 receptors are expressed in liver by differential transcription and processing of mRNA (9). Human FTF is identical to CPF variant 1 (10) and human CPF is identical to hB1F (10, 11). FTF and CPF differ in their N-terminal sequences (9, 10). It is possible that different amounts of NR5A2 isoforms may be expressed in different species and differentially regulate their target genes. It is also possible that FTF may compete with HNF4 for overlapping binding sites in the BARE-II. HNF4 has been established as an orphan receptor that is important for basal transcription of *CYP7A1* (8, 24).

Our results revealed that induction of SHP mRNA expression by bile acids in rat liver and HepG2 cells was much less than that observed in mice (16, 18). SHP expression is regulated mainly by monomeric nuclear receptors, such as FTF and steroidogenic factor 1 (25). The SHP induced by bile acids then feedback represses its own synthesis by interacting with FTF. It is therefore not surprising that bile acids and FXR do not induce SHP expression in rat livers and HepG2 cells. The relative expression levels of FXR, SHP, and FTF ultimately may determine both the *CYP7A1* and *SHP* expression levels in different species under different conditions. SHP functions primarily as a negative regulator that suppresses the activity of many nuclear receptors. Therefore, the level of SHP expression must be tightly regulated in vivo and the expression levels of SHP, HNF4, and FTF may determine the expression of cholesterol 7α-hydroxylase activity in the liver. The induction of SHP in mouse but not rat liver may explain the much lower CYP7A1 activity in mouse than rat livers. Other factors, such as bile acid pool size, composition and hydrophobicity, the efficiency of enterohepatic circulation of bile, and the relative expression levels of nuclear receptors involved in regulation of *CYP7A1* transcription, may explain the wide variations in cholesterol 7α-hydroxylase activity and mRNA expression levels in different species.

It has been suggested that SHP and FTF may regulate *CYP8B1* expression (16–18), because rat *CYP8B1* also has FTF-binding sites (26). We notice that the FTF-binding sites in the rat and human *CYP8B1* promoters also contain overlapping HNF4-binding sites, similar to the bile acid response element of the *CYP7A1* promoter. We propose that either FTF or HNF4 is involved in mediating bile acid repression depending on the structure of the bile acid response elements in the genes. **Figure 8** illustrates a cascade mechanism for FXR-mediated repression of *CYP7A1* transcription by bile acids, developed on the basis of this study. Bile acids bind and activate bile acid receptor, FXR. FXR then induces the expression of bile acid-responsive proteins such as FTF, SHP, and HNF4. When the SHP level increases, it interacts with FTF and represses its own transcription. In this mechanism, FTF could serve as a dominant negative factor that represses both *CYP7A1* and

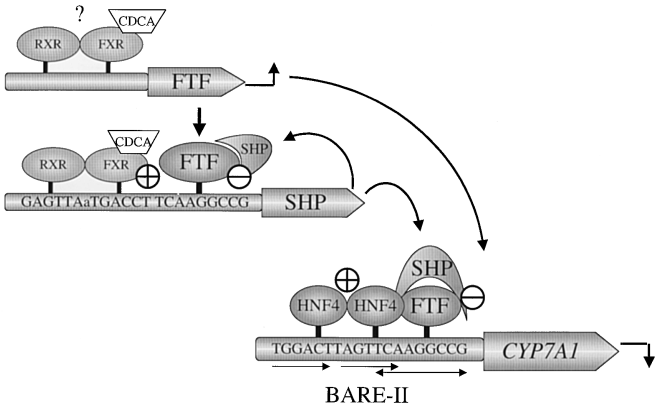

Fig. 8. Mechanism of FXR-mediated repression of *CYP7A1* transcription. When FXR/RXRα is activated by bile acid (BA), FXR/RXRα activates *FTF* transcription, but only moderately stimulate *SHP* transcription. FTF serves as a dominant negative factor in liver cells and inhibits both *SHP* and *CYP7A1* transcription. The FXR-binding site (IR1) in the SHP promoter is shown. The FXR-binding site in the FTF promoter has not been identified. SHP interacts with FTF and attenuates the inhibition of *SHP* and *CYP7A1* transcription. FTF may compete with HNF4 for binding to their overlapping binding sites in the BARE-II of the *CYP7A1* promoter.

SHP transcription. SHP interacts with either FTF or HNF4 and inhibits gene transcription depending on the structure of the genes. It is intriguing as why FXR, a relatively specific bile acid receptor, mediates its inhibitory effect through SHP and FTF, which are relatively nonspecific receptors. It seems that a cascade mechanism involving both bile acid receptor and bile acid-responsive protein not only amplifies the bile acid signals, but also confers the gene-specific and tissue-specific regulation of the genes in bile acid synthesis. How the *FTF* gene is regulated is not known at present. Identification of the FTF ligands, knockout of the *ftf* gene in mice, and cloning of the rat and human *FTF* genes would provide useful tools for studying *FTF* regulation.

Bile acid feedback regulation of the genes in bile acid synthesis plays an important role in maintaining cholesterol homeostasis. Bile acids may also regulate other genes in intermediate metabolism. The mechanisms of bile acid inhibition of gene transcription are complex and warrant further study to identify other target genes and nuclear receptors regulated by bile acids. These genes are potential targets for drug therapies to reduce serum cholesterol level and prevent cholestasis and cirrhosis.

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