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Transactivation of the Hepatitis B Virus Core Promoter by the Nuclear Receptor FXRα▽

Christophe Ramière,[1,2] Caroline Scholtès,[1,2] Olivier Diaz,[1] Vinca Icard,[1,2] Laure Perrin-Cocon,[1] Mary-Anne Trabaud,[2] Vincent Lotteau,[1,2] and Patrice André[1,2]*

INSERM U851, 21 Avenue Tony Garnier, and Université de Lyon, and Université Lyon 1, and IFR 128, Lyon F-69007, France,[1] and Hospices Civils de Lyon, Lyon F-69002, France[2]

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Hepatitis B virus (HBV) core promoter activity is positively and negatively regulated by nuclear receptors, a superfamily of ligand-activated transcription factors, via *cis*-acting sequences located in the viral genome. In this study, we investigated the role of farnesoid X receptor alpha (FXRα) in modulating transcription from the HBV core promoter. FXRα is a liver-enriched nuclear receptor activated by bile acids recognizing hormone response elements by forming heterodimers with retinoid X receptor alpha (RXRα). Electrophoretic mobility shift assays demonstrated that FXRα-RXRα heterodimers can bind two motifs on the HBV enhancer II and core promoter regions, presenting high homology to the consensus (AGGTCA) inverted repeat FXRα response elements. In transient transfection of the human hepatoma cell line Huh-7, bile acids enhanced the activity of a luciferase reporter containing the HBV enhancer II and core promoter sequences through FXRα. Moreover, using a greater-than-genome-length HBV construct, we showed that FXRα also increased synthesis of the viral pregenomic RNA and DNA replication intermediates. The data strongly suggest that FXRα is another member of the nuclear receptor superfamily implicated in the regulation of HBV core promoter activity and that bile acids could play an important role in the natural history of HBV infection.

Hepatitis B virus (HBV) infection is limited to hepatocytes of humans and primates and is associated with chronic hepatitis, cirrhosis, and hepatocellular carcinoma (10, 19). HBV is an enveloped virus containing a 3.2-kb partially double-stranded DNA genome with four open reading frames. These open reading frames encode the reverse transcriptase, precore, and core proteins; three surface antigen proteins (pre-S1, pre-S2, and S); and the X protein. Regulation of HBV transcription is under the control of four promoters (the core, pre-S1, pre-S2/S, and X promoters) and two enhancer regions (EN1 and EN2). For some authors, the core promoter corresponds to two distinct promoters, namely the precore and pregenomic promoters (44, 46). The core promoter activity, modulated by the EN2 region, plays a critical role in the virus life cycle. It initiates the synthesis of the precore and pregenomic 3.5-kb RNAs. The precore RNA encodes the precore protein, also called HBe antigen. The pregenomic RNA encodes both the polymerase and the core protein and serves as the template for viral DNA synthesis (24, 34).

Multiple cellular transcription factor binding sites have been identified on these regulatory sequences (26, 29, 31, 40, 48, 51), in particular for several nuclear receptors (NR) belonging to the superfamily of ligand-activated transcription factors (1, 11, 13, 14, 22, 23). Two important NR response elements (NRRE), situated in the EN2 ($NRRE_{enhII}$) and the core promoter ($NRRE_{pre\text{-}C}$) regions, are recognized by hepatocyte nuclear factor 4 alpha (HNF4α) and RXRα-peroxisome proliferator-activated receptor alpha (PPARα) heterodimers (13, 30, 44). In hepatoma cell lines, HNF4α and PPARα-RXRα upregulate synthesis of pregenomic RNA and viral DNA (43). Moreover, in nonhepatoma cells which do not support HBV replication, exogenous expression of these NR is sufficient for synthesis of the 3.5-kb RNA and viral replication intermediates (40). This observation demonstrated that liver-enriched NR play an essential role in the HBV life cycle and are likely to contribute to the restricted tropism of this virus.

In this study, the influence of farnesoid X receptor alpha (FXRα) on the transcriptional activity from the HBV core promoter was examined. FXRα is an NR activated by bile acids (25, 28) which is primarily expressed in the liver, intestine, and kidney. FXRα transactivates several genes implicated in the metabolism of bile acids and lipids, binding preferentially as a heterodimer with RXRα to regulatory sequences called FXRα response elements (FXREs). Typical FXREs are inverted repeats of two NR half-site sequences (5′-AGGTCA-3′) separated by 1 bp (IR-1 sequences) (7, 16). We identified two putative FXREs in the EN2 region and the core promoter. Using a reporter gene system, we demonstrated that FXRα transactivated the core promoter through both of these FXREs. Additionally, we observed that, in the context of a whole HBV genome, the global effect of FXRα was an increase in synthesis of the viral pregenomic RNA and DNA replication intermediates. Our results suggest that FXRα is another liver-enriched transcription factor implicated in the regulation of core promoter activity and level of viral replication.

MATERIALS AND METHODS

Materials. Chenodeoxycholic acid (CDCA), all-*trans*-retinoic acid (RA), and guggulsterone (GGS) were purchased from Sigma (Saint-Quentin Fallavier, France). Stock solutions were prepared with ethanol, chloroform, and dimethyl sulfoxide, respectively, diluted extemporaneously in complete Dulbecco's mod-

* Corresponding author. Mailing address: INSERM U851, 21 avenue Tony Garnier, Lyon F-69007, France. Phone: 33 4 37 28 23 27. Fax: 33 4 37 28 23 41. E-mail: patrice.andre@inserm.fr.

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ified Eagle's medium, and added to the cell cultures at the indicated concentrations.

Plasmids and oligonucleotides. Cloning of the plasmid pGL2-EN2/CP-wt was performed by standard techniques (32). A fragment of the HBV genome (GenBank accession number U95551) corresponding to nucleotides (nt) 1400 to 1902 was amplified from supernatants of HepG2.2.15 cells (35), using the following oligonucleotides: 5′-ATCGGAGCTCTGGATCCTGCGCGGGAC-3′ and 5′-ATCGAAGCTTGCCCCAAAGCCACCCAAG-3′. This fragment, which contained the EN2 and core promoter regions, was then inserted between the SacI and HindIII restriction sites into the pGL2 plasmid (Promega, Charbonnières, France) so that the expression of the firefly luciferase reporter gene was governed by the core promoter. Plasmids pGL2-EN2/CP-Em, pGL2-EN2/CP-Cm, and pGL2-EN2/CP-EmCm were created by PCR-based site-directed mutagenesis as previously described (50). The Em mutation converted the HBV sequence located between nt coordinates 1682 and 1694 from 5′-ATGTCAAC GACCG-3′ to 5′-AtaTCgACcACt-3′ (mutations are indicated in lowercase letters). The Cm mutation converted the sequence located between nt coordinates 1764 and 1776 from 5′-AGGTCTTTGTACT-3′ to 5′-cGcTaTTTaTACT-3′. All constructs were validated by sequencing (Cogenics Genome Express, Grenoble, France). The HBVx1.3 construct, which contains an overlength HBV genome of 4,195 bp ligated into plasmid pGEM-3Z, was a gift from Gilad Doitsh (Weizmann Institute of Science, Rehovot, Israel) and has previously been described (6). pSG5-FXRα and pSG5-RXRα, expression vectors for FXRα and RXRα, were gifts from Bart Staels (INSERM U545, Lille, France).

Cells and transfections. The human hepatoma cell line Huh7 was grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium containing 100 U/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 10% fetal bovine serum. Transfections for luciferase assays were performed with 24-well plates containing 1×10^5 cells per well with Lipofectamine 2000 (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's recommendations. The transfecting DNA mixture in each well comprised 200 ng of pGL2-EN2/CP-wt plasmid or its mutant derivatives, with 40 ng of pRL-SV40 plasmid serving as an internal control for transfection efficiency. pRL-SV40 (Promega) directs the expression of the *Renilla* luciferase gene under the control of the simian virus 40 (SV40) early enhancer/promoter. When appropriate, the DNA mixture included 200 ng of pSG5-FXRα and 100 ng of pSG5-RXRα. CDCA was used to activate FXRα. GGS, a natural FXRα antagonist, was used at 10 μM. At 3 days posttransfection, cells were lysed and assayed for luciferase activity by using a dual-luciferase reporter assay system (Promega). Values shown are averages of results for triplicate cultures.

Transfections for viral RNA and DNA analysis were performed in 10-cm plates containing 10^6 cells per well. Each well was transfected with 8 μg of the HBVx1.3 construct, with 200 ng of the pRL-SV40 plasmid as an internal control for transfection efficiency. In the cotransfection experiments, 2 μg of pSG5-FXRα and 1 μg of pSG5-RXRα were included. To keep the total DNA amount constant, the appropriate amount of pSG5 expression vector lacking the cDNA insert was added. CDCA and RA at 50 μM and 1 μM, respectively, were used to activate the NR FXRα and RXRα. GGS was used at 10 μM.

EMSA. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (18), with some modifications. Plasmids pSG5-FXRα and pSG5-RXRα were simultaneously in vitro transcribed and translated using a rabbit reticulocyte lysate according to the manufacturer's instructions (Promega). Synthetic oligonucleotides (Invitrogen) were annealed before being end labeled with [γ^{32}P]dATP (Perkin-Elmer, Courtaboeuf, France) or used as unlabeled competitors. The oligonucleotide pairs used to generate the FXR-CONS, FXR-EN2, and FXR-Cp double-stranded probes were 5′-GATCTCAAGAGG TCATTGACCTTTTTG-3′ and 5′-GATCCAAAAAGGTCAATGACCTCTTG A-3′ (FXR-CONS, which contains a consensus FXRE), 5′-GATCTCTGCAAT GTCAACGACCGACCTTGA-3′ and 5′-GATCTCAAGGTCGGTCGTTGAC ATTCAGA-3′ (FXR-EN2, HBV coordinates 1682 to 1694), and 5′-GATCGA TTAGATTAAAGGTCTTTGTACTAGGA-3′ and 5′-GATCTCCTAGACAA AGACCTTTAATCTAATC-3′ (FXR-Cp, HBV coordinates 1764 to 1776). ^{32}P-labeled double-stranded oligonucleotides (1 ng) were incubated at room temperature for 20 min with 2 μl of reticulocytes lysates containing or not containing the translated protein factors, 5 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 15% glycerol), 2 μg of poly(dI-dC)–poly(dI-dC), and water to obtain a final volume of 25 μl. The resulting complexes were then separated on a native 4% polyacrylamide gel in 0.5× Tris-glycine-EDTA at 50 V. After electrophoresis, the EMSA complex was visualized by autoradiography. When competition analysis was performed, 200 ng of a cold double-stranded competitor oligonucleotide or 1 μl of antibody was added to the reticulocyte lysate 15 min prior to the addition of the ^{32}P-labeled double-stranded oligonucleotide. FXRα and RXRα antibodies (SC-13063 and SC-553, respectively) were purchased from Santa Cruz Biotechnology. The irrelevant antibody used was mouse anti-hepatitis C virus core antigen antibody C-830. The oligonucleotide pair used to generate the double-stranded irrelevant DNA probe was 5′-GATCCTTCTGGGAATTCCTAGATC-3′ and 5′-GATCT AGGAATTCCCAGAAGGATC-3′.

Analysis of viral RNA and DNA. At 3 days posttransfection, cells were divided equally and harvested for viral RNA and DNA analysis. Total cellular RNA was isolated using an RNeasy kit (Qiagen) and quantified by determining the absorbance at 260 nm. Northern and Southern blot analyses were performed as described previously (32). For Northern blot analysis, 2 μg of RNA per sample was separated on a 1% agarose-formaldehyde gel and blotted to a nylon membrane (Roche). For Southern blot analysis, viral DNA replication intermediates were isolated as previously described (40), subjected to electrophoresis on a 1% agarose–Tris-borate-EDTA gel, and transferred to a nylon membrane. The membranes were then hybridized with a radiolabeled HBV probe. For viral RNA analysis, the blot was then stripped and reprobed with a *Renilla* luciferase probe. The HBV radioactive probe was prepared by using the DNA of the X and core gene regions. For the *Renilla* luciferase DNA probe, a 1-kb fragment of plasmid pRL-SV40 was used. Probes were labeled using a random-prime labeling system (Promega) with [α-^{32}P]dATP (3,000 Ci/mmol; Perkin Elmer). About 10^6 cpm labeled probes were used per 1 ml of hybridization buffer. Densitometry was performed with a PhosphorImager.

Primer extension analysis was performed with 5 μg of total cellular RNA per sample by using a primer extension system (Promega) according to the manufacturer's recommendations. The primer used to determine the relative amounts of the precore and pregenomic RNAs was 5′-GAGAGTAACTCCACAGTA T-3′ (46). The primer used to analyze the *Renilla* luciferase transcript was 5-GAAGAGGCCGCGTTACCATG-3′.

RESULTS

Identification of FXRα binding sites on the core promoter and enhancer II regions. The consensus FXRE was described as an IR-1 sequence, 5′-AGGTCANTGACCT-3′ (17). We observed that the HBV EN2 and core promoter regions contained two sequence elements with high homology to this consensus FXRE, corresponding to HBV nt coordinates 1682 to 1694 (in the EN2 region) and HBV nt coordinates 1764 to 1776 (in the core promoter). It is noteworthy that the sequence element identified in the core promoter overlaps $NRRE_{pre\text{-}C}$, a binding site for the NR PPARα-RXRα, HNF4α, and chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) (Fig. 1).

EMSAs were performed with two radiolabeled double-stranded oligonucleotides, FXR-EN2 and FXR-Cp, corresponding to the putative FXREs identified in the EN2 and the core promoter regions, respectively. The binding of FXRα and RXRα recombinant polypeptides to FXR-EN2 and FXR-Cp sequences was compared to that observed for a perfect inverted repeat sequence (FXR-CONS). The RXRα polypeptide did not bind the FXR-CONS sequence, whereas two complexes were formed with the FXRα polypeptide, representing FXRα binding either as a monomer or as homodimers (Fig. 2A). In the presence of both polypeptides, a unique band was observed, representing binding of FXRα-RXRα heterodimers to the FXR-CONS sequence, as expected (4). With FXRα or RXRα polypeptides alone, a complex was formed with either the FXR-EN2 or the FXR-Cp sequence (Fig. 2B and C). This complex, which was still observed in the presence of both NR, probably represented binding of FXRα and RXRα monomers to FXR-EN2 and FXR-Cp sequences. Similar binding of NR monomers to specific sequences of the HBV genome has previously been described, in particular binding of PPARα monomers to $NRRE_{pre\text{-}C}$ in the presence or absence of RXRα (30). In the presence of both FXRα and RXRα polypeptides, a

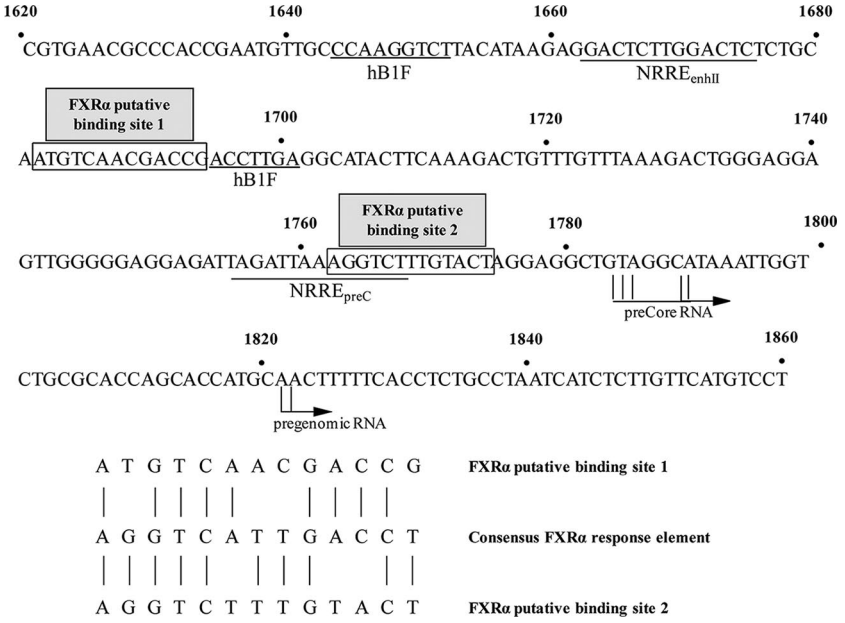


FIG. 1. Nucleotide sequences of the HBV enhancer II and core promoter regions. The nucleotide coordinates are derived from the GenBank database (HBV genome, subtype ayw; GenBank accession number U95551). The underlined sequences correspond to the previously described response elements NRRE$_{enhII}$ (a binding site for HNF4α) and NRRE$_{pre\text{-}C}$ (a binding site for PPARα/RXRα, HNF4α, COUP-TF1, and the testicular receptor TR4) and two binding sites for the HBV enhancer II B1 binding factor hB1F. The arrows indicate the initiation sites of synthesis of the precore and pregenomic RNAs. The two putative FXREs are represented by boxes. For each sequence, the homology with the consensus FXRE is indicated.

slower-migrating complex was observed. This complex was not observed when competition analyses were performed with either the double-stranded oligonucleotide probe containing the consensus FXRE (FXR-CONS) or antibodies directed against the DNA-binding site of FXRα or RXRα. These results indicate that the slower-migrating complex represents binding of FXRα-RXRα heterodimers to FXR-EN2 and FXR-Cp sequences. The affinity of these heterodimeric complexes seems to be higher for the FXR-EN2 sequence than for the FXR-Cp sequence.

FXRα activates transcription from the HBV core promoter. To examine the functional properties of the FXRα binding

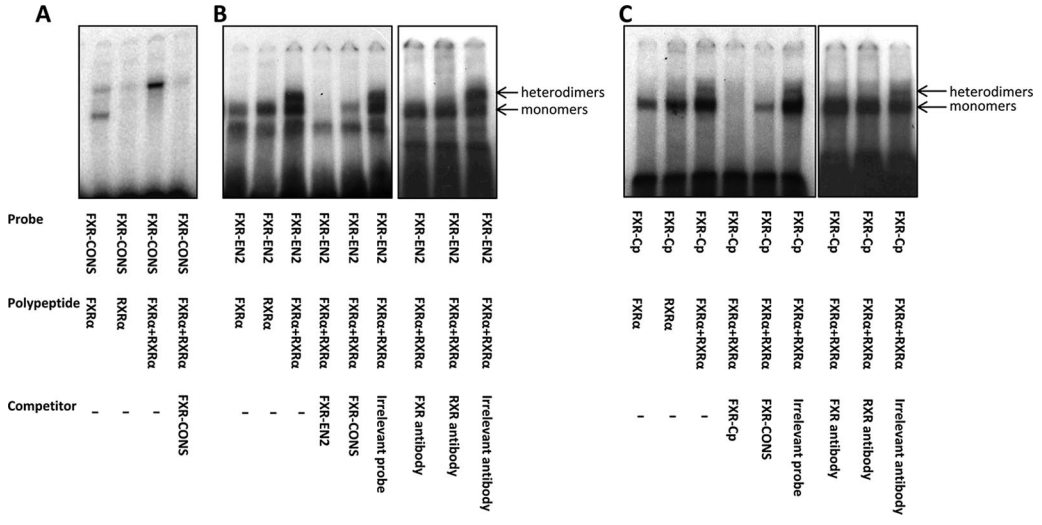


FIG. 2. Identification of two FXREs in the HBV enhancer II and core promoter regions. EMSAs were performed with in vitro-expressed FXRα and RXRα polypeptides. Binding of the recombinant NR was analyzed with ^{32}P-labeled double-stranded oligonucleotides FXR-CONS, which contains a consensus FXRE (A); FXR-EN2, which contains the putative FXRE located in the EN2 region of HBV (B); and FXR-Cp, which contains the putative FXRE located in the HBV core promoter (C). The specificity of the binding to the probes was verified using unlabeled double-stranded oligonucleotides as competitors or antibodies directed against FXRα and RXRα. The arrows indicate the positions of the DNA-protein complexes.

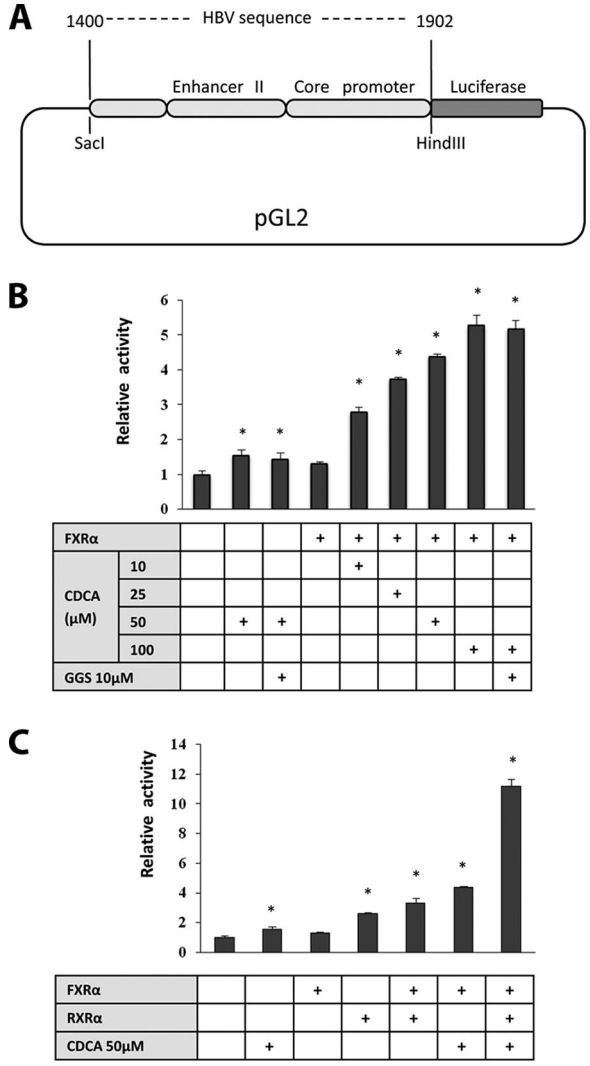


FIG. 3. FXRα activates the transcription from the HBV core promoter in Huh-7 cells. (A) Schematic representation of the firefly luciferase reporter plasmid pGL2-EN2/PC-wt. The fragment of the HBV genome comprised between nt 1400 and 1902, which contains the enhancer II and core promoter regions, was cloned between the SacI and HindIII restriction sites of pGL2. Huh-7 cells were cotransfected with pGL2-EN2/PC-wt; pRL-SV40, which contains a *Renilla* luciferase gene; and expression vectors of FXRα alone (B) or FXRα and RXRα (C). CDCA and GGS, the FXRα ligands, were added at the indicated concentrations. At 3 days posttransfection, cells were harvested to determine firefly luciferase activity. The results were normalized for transfection efficiency with *Renilla* luciferase activity and expressed as differences (*n*-fold) from the control level. The standard deviations of the means are indicated by error bars. Asterisks indicate statistically significant increases in relative activity, as determined by Student's *t* test ($P < 0.05$).

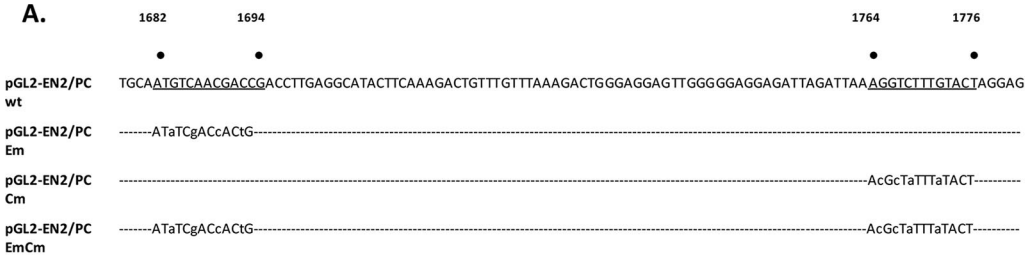
sites identified in the core promoter and the EN2 region, we performed cotransfections of an expression vector for FXRα together with the plasmid pGL2-EN2/PC-wt, a luciferase reporter construct containing HBV nt 1400 to 1902, in the hepatoma cell line Huh-7 (Fig. 3A). CDCA, a potent FXRα activator bile acid, was added at concentrations ranging from 10 μM (which corresponds to normal seric concentrations) to 100 μM (which is observed in cholestatic hepatitis) (8). We first verified that FXRα and bile acids did not influence transcription of the *Renilla* luciferase gene from the plasmid pRL-SV40, which was used to normalize the results for transfection efficiency (data not shown). Expression of FXRα alone did not significantly modify core promoter activity (Fig. 3B). Addition of 50 μM CDCA in the absence of exogenous FXRα slightly increased the level of transcription of the core promoter, which likely reflects the endogenous expression of FXRα in Huh-7 cells (data not shown). Expression of FXRα in the presence of various concentrations of CDCA increased the level of transcription from the core promoter from three- to fivefold, depending on the concentration of CDCA. These results indicate that FXRα activates transcription from the core promoter in a ligand-dependent manner.

We next examined the effect of GGS on core promoter activity. GGS is a natural FXRα antagonist extracted from the plant *Commiphora mukul*. GGS inhibits FXRα activation by bile acids at the micromolar range (42). However, in our experiments, a concentration of 10 μM GGS did not inhibit the CDCA-induced core promoter activation with or without overexpression of FXRα.

FXRα binds preferentially to its response elements as a heterodimer with RXRα (16). Thus, we examined the influence of both FXRα and RXRα on core promoter activity. Expression of RXRα alone slightly increased transcription from the core promoter, whereas a threefold increase was observed with exogenous expression of FXRα and RXRα in the absence of CDCA (Fig. 3C). Maximal induction of the transcriptional activity of the core promoter was observed when both NR were expressed in the presence of bile acids. A >10-fold increase of core promoter activity was measured with 50 μM CDCA in the presence of FXRα and RXRα. Therefore, it appears that FXRα and RXRα synergistically activate the core promoter.

FXRα transactivates the HBV core promoter through both FXRα binding site 1 and FXRα binding site 2. To determine whether FXRα activates the core promoter by directly interacting with the HBV genome, we performed mutational analysis of the two FXREs identified in the EN2 and the core promoter regions. Several point mutations were introduced into FXRα binding site 1, located in the EN2 region (plasmid pGL2-EN2/PC-Em); FXRα binding site 2, located in the core promoter (plasmid pGL2-EN2/PC-Cm); or both sites (plasmid pGL2-EN2/PC-EmCm) (Fig. 4A). We next examined the effects of these mutations on the FXRα-mediated activation of the core promoter in transient-transfection experiments with Huh-7 cells. When FXRα binding site 2 was mutated, the basal activity of the core promoter was severely decreased (Fig. 4B). This result was not surprising, as this sequence overlaps $NRRE_{pre\text{-}C}$, an essential determinant of core promoter activity. When FXRα binding site 1 was mutated, transcription from the core promoter was also significantly impaired, suggesting that this sequence is also essential for the proper functioning of the core promoter. However, in each case, FXRα was still able to enhance transcription from the core promoter, but with a much lesser efficacy than the wild-type sequence. Thus, overexpression of FXRα plus CDCA led to a 3-fold increase in the presence of a mutant of FXRα binding site 1 and a 4.2-fold increase in the presence of a mutant of FXRα binding site 2, compared to a >10-fold increase observed with the wild-type

A.



B.

	Relative luciferase activity				Fold induction		
	Mock	+ CDCA	+ FXR	+ FXR + CDCA	+ CDCA	+ FXR	+ FXR + CDCA
pGL2-EN2/PC wt	**1.00** (0.68)	**3.43** (1.15)	**2.65** (0.40)	**11.40** (1.91)	**3.43**	**2.65**	**11.40**
pGL2-EN2/PC Em	**0.12** (0.04)	**0.27** (0.03)	**0.23** (0.04)	**0.36** (0.06)	**2.20**	**1.92**	**2.95**
pGL2-EN2/PC Cm	**0.08** (0.04)	**0.17** (0.08)	**0.17** (0.02)	**0.34** (0.02)	**2.05**	**2.12**	**4.18**
pGL2-EN2/PC EmCm	**0.08** (0.02)	**0.08** (0.02)	**0.12** (0.05)	**0.12** (0.04)	**0.97**	**1.53**	**1.59**

FIG. 4. Mutational analysis of the FXREs located in the EN2 and core promoter regions of HBV. (A) Partial sequence of the construct pGL2-EN2/PC-wt showing the wild-type EN2 and core promoter regions of HBV from nt 1678 to nt 1782. FXRα binding sites are underlined. For each mutant construct (pGL2-EN2/PC-Em, Cm, and EmCm), mutations introduced into FXRα binding sites 1 and 2 are indicated in lowercase letters, whereas unchanged nucleotides are indicated in capital letters. (B) Relative activities of the wild-type and mutant constructs in Huh-7 cells, with or without exogenously expressed FXRα in the presence or absence of CDCA. Transfection was performed as described in the legend to Fig. 3. CDCA was added at 100 μM. The transcriptional activities are reported relative to the activity of the pGL2-EN2/PC-wt construct in the absence of exogenously expressed FXRα and CDCA. The results were normalized for transfection efficiency with *Renilla* luciferase activity. The standard deviations of the means are indicated in parentheses.

sequence. When both site 1 and site 2 were mutated, the FXRα-mediated activation of the core promoter was nearly completely abolished. Together, these results indicate that FXRα transactivates the HBV core promoter through both FXRα binding site 1 and FXRα binding site 2, located in the EN2 and the core promoter regions.

Effects of FXRα and RXRα on viral RNA synthesis. Transcription of the 3.5-kb HBV RNA from the core promoter is modulated by the EN2 region. However, in the context of a whole HBV genome, the EN2 region is not autonomous but dependent on an active EN1 region (6). Moreover, it has previously been shown that transcription factor HNF3 increased transcription from the core promoter by using reporter gene constructs (15, 20) but inhibited pregenomic RNA synthesis in the context of a greater-than-genome-length HBV construct (39, 40). Thus, we next determined if the FXRα-mediated activation of the core promoter that we observed with a luciferase reporter construct lacking the EN1 region correlated with an increase of the viral 3.5-kb RNA synthesis. The influence of FXRα and RXRα on viral RNA synthesis was evaluated by transient transfection of Huh-7 cells with a 1.3xHBV DNA construct encoding all viral transcripts (6). Exogenous expression of FXRα alone did not influence the synthesis of the 3.5-kb HBV RNA (Fig. 5), but a twofold increase was observed in the presence of 50 μM CDCA. The same twofold increase was also observed when the respective ligands of FXRα and RXRα, i.e., CDCA and RA, were added without exogenous expression of the NR. As RXRα and FXRα are expressed in Huh-7 cells (data not shown), activation of endogenous RXRα and FXRα may explain this result. Maximal induction of the 3.5-kb transcript synthesis was observed with exogenous expression of both FXRα and RXRα in the presence of CDCA and RA, which induced a nearly threefold increase in the 3.5-kb RNA synthesis. GGS did not significantly modify synthesis of the 3.5-kb RNA.

Two other HBV transcripts were also visualized. Synthesis of the 0.7- and 2.1-kb RNAs is regulated by the X promoter and the pre-S2/S promoter, respectively. FXRα did not modulate synthesis of the 0.7-kb RNA, whereas a moderate increase in the transcription of the 2.1-kb RNA was observed with overexpression of both FXRα and RXRα and addition of their ligands. The minor 2.4-kb viral transcript, whose synthesis is regulated by the pre-S1 promoter, was not visualized in our experiments.

FXRα increases synthesis of the pregenomic RNA and viral DNA. It has previously been demonstrated that transcription of the precore and pregenomic RNAs can be differentially regulated by NR and that the HBV core promoter may actually correspond to two distinct overlapping promoters (41, 43–46). The precore and the pregenomic RNAs exert opposite effects on viral replication. The pregenomic RNA serves as the template for viral DNA synthesis, whereas the precore RNA encodes the HBe antigen, which downregulates the viral replication. Thus, we next analyzed by primer extension assays whether FXRα modified the relative amounts of these transcripts. Synthesis of the pregenomic RNA was increased 2.6-

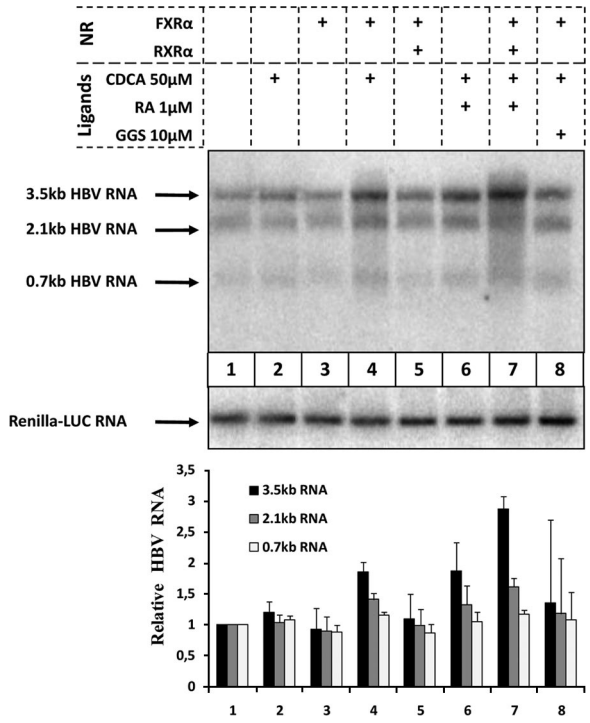


FIG. 5. FXRα enhances synthesis of the HBV 3.5-kb RNA. Huh-7 cells were transiently transfected with a 4.2-kb HBV DNA construct together with FXRα and RXRα expression vectors. CDCA, GGS, and RA, the FXRα and RXRα ligands, were added at the indicated concentrations. Cells were harvested at 3 days posttransfection for total RNA analysis. (A) Northern blot analysis of HBV transcripts was performed with an HBV probe corresponding to the X and core regions. Arrows indicate the locations of the 3.5-, 2.1-, and 0.7-kb transcripts. Hybridization of the *Renilla*-LUC transcript was used as an internal control for transfection efficiency. (B) Quantitative analysis of the 3.5-kb, 2.1-kb, and 0.7-kb HBV RNAs determined with a PhosphorImager. The mean RNA levels plus standard deviations from two independent experiments are represented. Results for densitometry are expressed relative to the amounts of the RNAs under control conditions (lane 1).

fold when FXRα was exogenously expressed in the presence of CDCA and 3.6-fold when RXRα was also overexpressed (Fig. 6A and C). Synthesis of the precore RNA was enhanced to a lesser extent by FXRα, with a 1.7-fold increase in the presence of FXRα plus CDCA and a 2.4-fold increase with both FXRα and RXRα overexpressed in the presence of their ligands.

To examine the global effect of FXRα on viral DNA synthesis, Southern blot analysis of HBV DNA accumulated in the cytoplasm of transfected cells was performed. Exogenous expression of FXRα in the presence of CDCA resulted in a nearly fourfold increase in viral DNA synthesis (Fig. 6B and D). This result correlated with the FXRα-induced modification of the ratio between the pregenomic and precore transcripts. Thus, we conclude that FXRα can significantly enhance the level of HBV replication, likely through a preferential activation of the pregenomic RNA synthesis.

DISCUSSION

In this study, we identified two putative FXREs in the HBV genome with homology to the typical inverted repeat sequence recognized by FXRα. The first FXRE is located in the EN2 region, between HBV nt 1682 and 1694, and is recognized by FXRα-RXRα heterodimers. To our knowledge, this sequence has not previously been described as a response element for other NR. Analysis of different HBV genomes in the GenBank database reveals that this sequence is well conserved among genotypes A, B, C, D, E, and G. Interestingly, the nucleotide sequences are somewhat different in genotypes F (5′-CGGTC AATGACCT-3′) and H (5′-CGGTCAACGACCT-3′) and present even higher homologies to the consensus FXRE. Differences in transcriptional regulation by COUP-TF1 have recently been reported to occur among HBV genotypes (9). Similarly, the influence of FXRα on different HBV strains could be further explored. The second FXRE is located in the core promoter, between nt 1764 and 1776, and can also bind FXRα-RXRα heterodimers. Interpretation concerning this second putative FXRE is rendered difficult as this sequence overlaps the well-characterized response element $NRRE_{pre\text{-}C}$, a direct repeat (DR-1) sequence recognized by PPARα/RXRα and HNF4α, whose role in the modulation of core promoter activity is critical. Though FXRα interacts preferentially with IR-1 sequences, the capacity of this NR to bind to DR-1 sequences has also been demonstrated, although with a weaker affinity (17). This suggests that a competition could exist between PPARα, RXRα, HNF4α, and FXRα for binding to this *cis*-acting region of the HBV genome.

The functional significance of these two FXREs was investigated by transient transfection in the hepatoma cell line Huh-7, demonstrating that bile acids, through FXRα, activate the HBV core promoter. This effect was modulated by exogenous expression of RXRα, the FXRα preferential transactivation partner. Mutational analysis of FXRα binding sites 1 and 2 confirmed that FXRα-induced activation of the core promoter was mediated through both FXREs. Whereas EMSA experiments suggested that the affinity of FXRα-RXRα heterodimers was higher for the FXRE located in the EN2 region, results for the functional analysis did not reveal a significantly predominant role for either site in FXRα-induced activation of the core promoter. We cannot exclude that other FXRα binding sites exist in the HBV genome. However, results for the mutational analysis suggest that the two FXREs identified in this study are the principal *cis*-acting sequences responsible for the FXRα-induced activation of the HBV core promoter.

A recent study showed that the small heterodimer partner (SHP) whose expression is under the control of FXRα could inhibit HBV replication in the HepG2 cell line, likely through a direct interaction with HNF4α (27). However, using a greater-than-genome-length construct, we showed that the global effect of FXRα activation by bile acids, despite the induction of SHP expression in Huh-7 cells (data not shown), is an increased transcription of the HBV pregenomic RNA and, consequently, an increase in the level of viral replication. This suggests that the effect of FXRα on HBV transcription primarily depends on its direct binding to the FXREs and that the FXRα-induced expression of SHP is not sufficient to counterbalance the global activation of the core promoter. Northern blot analysis also revealed that FXRα does not influence the synthesis of the 0.7-kb RNA but slightly increases the synthesis of the 2.1-kb RNA. These results are in agreement with previous work demonstrating that transcription from the X pro-

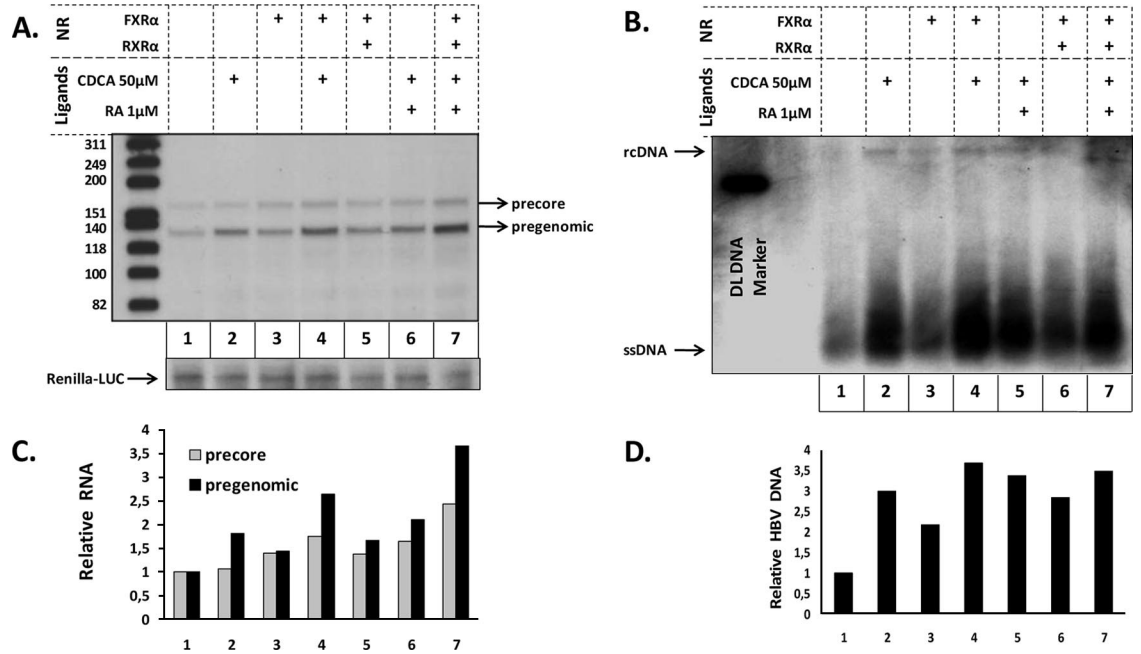


FIG. 6. FXRα enhances synthesis of the pregenomic RNA and viral DNA. Huh-7 cells were transfected as described in the legend to Fig. 5. At 3 days posttransfection, cells were divided equally for extraction of total cellular RNA or viral replication intermediates. For each condition, 5 μg of total RNA was used for primer extension analysis of the relative amounts of precore and pregenomic RNAs (A). Detection of the *Renilla* luciferase transcript served as an internal control for transfection efficiency. ^{32}P-labeled DNA fragments were used as size markers. The lengths of base pairs for each fragment in the marker lane are indicated next to the corresponding band. The expected sizes for the primer extension products were 175 bp for the precore RNA and 135 bp for the pregenomic RNA. For Southern blot analysis of viral replication intermediates (B), hybridization was performed with the same HBV DNA probe used in the Northern blot. The positions of the viral DNAs are indicated by arrows. A duplex linear HBV DNA (DL DNA) was used as a control and size marker. rcDNA, relaxed circular DNA; ssDNA, single-strand DNA. Quantitative analyses of the precore and pregenomic RNAs (C) and viral DNA (D) were performed with a PhosphorImager. Results for densitometry are expressed relative to the levels for the control conditions (lane 1).

moter is essentially under the control of EN1 (6) whereas transcription from the pre-S2/S promoter is also modulated by the EN2 region (47).

Following the demonstration of the essential role of NR in the HBV life cycle, NR agonists and antagonists were envisaged as possible new therapeutic targets against HBV. Bile acids were recently shown to enhance genotype 1 hepatitis C virus replication in a subgenomic system, and GGS, an FXRα antagonist, significantly inhibited this effect (33). However, in our study, GGS showed little or no effect in inhibiting core promoter activity or viral RNA synthesis. This observation may be explained by the fact that GGS, after being described as a pure FXRα antagonist, is now envisioned as a gene-selective FXRα modulator. For instance, GGS has been shown to enhance the expression of the bile salt export pump, one of the target genes for FXRα (5). As its antagonist effect seems to be dependent, at least partly, on the nucleotide sequences of the FXREs, it will be interesting to examine the influence of GGS on different HBV genotypes or test other synthetic FXRα modulators.

Several studies have described in detail the essential role of NR PPARα-RXRα and HNF4α in the HBV life cycle (30, 40, 43–45). The relative influence of FXRα compared to that of these NR remains to be clarified, but our results strongly suggest that FXRα may also contribute to the modulation of the HBV core promoter activity in vivo and subsequently to the level of viral replication. Interestingly, in HBV transgenic mice, synthesis of the 3.5-kb RNA greatly predominates in hepatocytes and proximal convoluted renal tubules (12). The liver and kidney express not only NR PPARα and HNF4α but also FXRα (38). Thus, FXRα could be another factor implicated in the HBV-restricted tropism at the transcriptional level.

Shlomai and Shaul showed recently that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a coactivator of several transcription factors controlling energy and nutrient homeostasis, coactivates HBV replication after short-term fasting in mice (36). Moreover, this process was at least partly dependent on HNF4α. Subsequently, they proposed the concept of "metabolovirus" to describe the regulation of HBV replication (37). In the metabolovirus model, the regulation of HBV transcription is modulated by nutritional signals and is similar to what was found for hepatic metabolic genes, such as the PEPCK and G6Pase genes, both implicated in gluconeogenesis. Upon starvation, PGC-1α is induced and coactivates HNF4α, resulting in the upregulation of HBV replication. Interestingly, PGC-1α is also known to coactivate FXRα and stimulate the expression of FXRα target genes. During the response to short-term fasting, whereas PPARα and HNF4α stimulate the beta-oxidation and gluconeogenesis pathways, respectively (21), FXRα is supposed to participate in this metabolic response by decreasing triglyceride production and secretion (49) and also by modulating gluconeogenesis (2). Based on the metabolovirus model, we suggest that bile acids,

through FXRα, are implicated in the metabolic regulation of HBV transcription.

To our knowledge, the only work that examined in vivo the relations between bile acids and viral hepatitis focused on ursodeoxycholic acid (3), which is a very weak FXRα activator. In our experiments, bile acids increased HBV core promoter activity in a dose-dependent manner. A modest but significant effect was measured with 10 μM CDCA, which corresponds to normal seric levels. Concentrations leading to the maximal induction, i.e., 50 μM and 100 μM CDCA, are observed only in cholestatic hepatitis and cirrhosis (8). Possible correlations in vivo between bile acid concentrations and the natural history of HBV infection, in terms of risk of chronicity, severity of evolution, occurrence of exacerbation episodes, and prognosis, should be further examined to evaluate the potential benefit of therapeutics such as FXRα modulators or bile acid sequestrants.

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