JOURNAL OF VIROLOGY, Dec. 1997, p. 9366–9374 Vol. 71, No. 12
0022-538X/97/$04.00+0

Differential Regulation of the Pre-C and Pregenomic Promoters of Human Hepatitis B Virus by Members of the Nuclear Receptor Superfamily

XIANMING YU AND JANET E. MERTZ*

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706-1599

Received 20 May 1997/Accepted 10 September 1997

Synthesis of the pre-C and pregenomic RNAs of human hepatitis B virus (HBV) is directed by two overlapping yet separate promoters (X. Yu and J. E. Mertz, J. Virol. 70:8719–8726, 1996). Previously, we reported the identification of a binding site for the nuclear receptor hepatocyte nuclear factor 4 (HNF4) spanning the TATA box-like sequence of the pre-C promoter. This HNF4-binding site consists of an imperfect direct repeat of the consensus half-site sequence 5′-AGGTCA-3′ separated by one nucleotide; i.e., it is a DR1 hormone response element (HRE). We show here that other receptors, including chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1), human testicular receptor 2 (TR2), and peroxisome proliferator-activated receptors (PPARs) as heterodimers with retinoid X receptors (RXRs), can also specifically bind this DR1 HRE. Synthesis of the pre-C and pregenomic RNAs was affected both in transfected hepatoma cells and in a cell-free transcription system by the binding of factors to this DR1 HRE. Interestingly, whereas some members of the hormone receptor superfamily differentially repressed synthesis of the pre-C RNA (e.g., HNF4 and TR2) or activated synthesis of the pregenomic RNA (e.g., PPARγ-RXRα), other members (e.g., COUP-TF1) coordinately repressed synthesis of both the pre-C and pregenomic RNAs. Thus, HBV likely regulates its expression and replication in part via this DR1 HRE. These findings indicate that appropriate ligands to nuclear receptors may be useful in the treatment of HBV infection.

Human hepatitis B virus (HBV) is a small DNA virus with a marked hepatotropism. Its 3.2-kb DNA genome contains four overlapping open reading frames which encode the surface antigen proteins (large S, middle S, and major S), core antigen proteins (precore and C), reverse transcriptase (P), and X protein. Replication of the HBV genome occurs through an RNA intermediate. The longer than genome-size pregenomic RNA is encapsidated along with P protein into core particles. During maturation of the core particles, the pregenomic RNA is reverse transcribed and converted into partially double stranded DNA. The mature core particles acquire an envelope containing lipid and surface antigen proteins while budding into the endoplasmic reticulum. HBV virion particles are transported by a vesicle transport pathway and secreted from the cells. The coordinate expression of the genes of HBV is achieved by regulation of HBV's promoters through two enhancer regions and other *cis*-acting promoter elements (Fig. 1) (reviewed in references 14 and 15).

Patients chronically infected with HBV are at high risk for developing hepatocellular carcinoma (HCC). The incidence of HCC in HBV-infected individuals is severalfold higher in males than in females (1, 46). This fact has been attributed to hormones playing general roles in the development of HCC. However, hormones may also play important roles in hepatocarcinogenesis through their direct involvement in the regulation of expression of the HBV genome in HBV-infected individuals. Tur-Kaspa et al. (50, 51) identified a glucocorticoid response element situated upstream of the enhancer I region of the HBV genome. Recently, several additional hormone response elements (HRE) have been identified in the HBV genome (Fig. 1) (16, 18, 21, 22, 40, 55).

HREs function via the binding of members of the nuclear receptor superfamily (34, 35). Frequently, different nuclear receptors can compete for binding to the same HRE (48). For example, the nuclear receptors hepatocyte nuclear factor 4 (HNF4), retinoid X receptor α (RXRα), chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1), and peroxisome proliferator-activated receptor α (PPARα) can all bind to the same recently identified HRE in HBV enhancer I (named the GB element). However, they have distinct effects on enhancer I activity in hepatoma cell lines: whereas HNF4, RXRα, and PPARα-RXRα heterodimers activate transcription from a promoter-reporter construct linked to the GB element, COUP-TF1 represses transcription from this same promoter construct (16, 21, 22, 40). Thus, the activities of HBV's promoters are likely modulated in part through the binding of different nuclear receptors to HREs.

We previously reported that synthesis of the pre-C and pregenomic RNAs is regulated by two separate promoters (Fig. 1). We showed that expression of these two promoters can be differentially regulated by ubiquitous (e.g., Sp1) and liver-enriched (e.g., HNF4) transcription factors and identified a novel HNF4-binding site, called the DR1 HRE, spanning the TATA box-like sequence of the pre-C promoter (55). We report here the identification of additional nuclear receptors that can bind this HRE and the effects of their binding on synthesis of the pre-C and pregenomic RNAs.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Plasmid pWT contains nucleotides (nt) 1403 to 1991 of the HBV genome (subtype adr) cloned into the *Bam*HI site of plasmid pGL2 (Promega). Plasmid pMinser is a derivative of pWT containing a 15-bp insertion between HBV nt 1789 and 1790 (Fig. 1). Construction of pWT and pMinser was reported previously (55). Plasmid pDR1$^-$, which contains two G-to-C point mutations in the DR1 HRE (Fig. 1), was derived from pWT by PCR-based mutagenesis (4). Plasmids pCOUP-TF1 and pRSV-COUP-TF1 (9) (gifts from M.-J. Tsai), encoding human COUP-TF1, were used for synthesis of recombinant COUP-TF1 by cell-free transcription and translation and in transient transfection, respectively. Plasmid pSG5-TR2 (30) (a gift from C. Chang)

* Corresponding author. Phone: (608) 262-2383. Fax: (608) 262-2824. E-mail: mertz@oncology.wisc.edu.

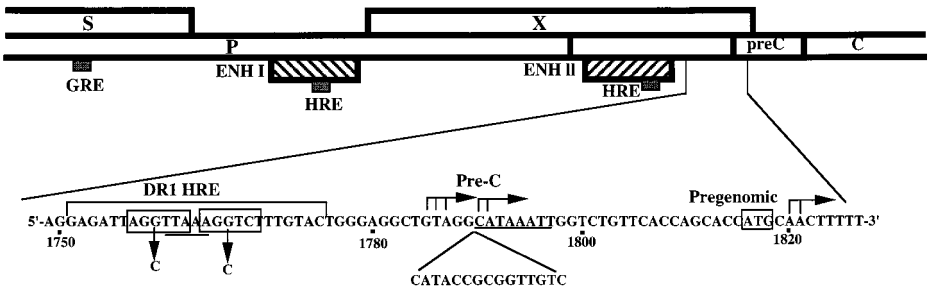

FIG. 1. Schematic diagram of the HBV pre-C and pregenomic promoters and their locations within the HBV genome. Portions of the P, S, pre-C, C, and X open reading frames are indicated by open rectangular boxes. Enhancers (ENH) I and II are indicated by hatched rectangular boxes. The HREs and glucocorticoid response element (GRE) are indicated by small shaded squares. The nucleotide sequence of the pre-C/pregenomic promoter region is shown. The horizontal arrows indicate the locations of the 5′ ends of the pre-C and pregenomic RNAs. The TATA box-like sequences for the two promoters are underlined. The translation initiation codon of the pre-C open reading frame is indicated by a box. The two half-sites of the DR1 HRE are also boxed, with the two G-to-C mutations in DR1$^-$ indicated by the downward arrows. The location and sequence of the 15-bp insertion in mutant pMinser is indicated below the WT promoter sequence. The DR1 oligonucleotide used in GMSAs and cell-free transcriptions is indicated by a bracket.

was used for synthesis of human testicular receptor 2 (TR2). Plasmid pT7-PPARα (6) (a gift from D. Moore) was used for synthesis of recombinant PPARα. Plasmids pSGhRXRα (31) and pSV-SPORT-PPARγ2 (47) (gifts from P. Chambon and M. Spiegelman, respectively) were used for synthesis of recombinant RXRα and PPARγ both in vitro and in transiently transfected cells. Plasmid pCDMHNF4 encodes recombinant rat HNF4 and has been described previously (55). Plasmid pEQ176 encodes β-galactosidase (β-Gal) (43).

Synthetic oligonucleotides (Integrated DNA Technologies, Inc.) were annealed and gel purified before radiolabeling or use as unlabeled competitors. The double-stranded oligonucleotide DR1 is a 25-mer (HBV nt 1751 to 1775) containing the DR1 HRE; the double-stranded oligonucleotide DR1$^-$ is the same as DR1 except for two G-to-C point mutations (Fig. 1). The double-stranded oligonucleotide SV40 +55, corresponding to the +55 region of the simian virus 40 (SV40) major late promoter, is a 22-mer (5′-GTTAAGGTTCG TAGGTCATGGA-3′) which contains an imperfect DR2 HRE (57).

GMSAs. Gel mobility shift assays (GMSAs) and immunoshift assays were performed as described previously (52, 57, 58). The recombinant proteins COUP-TF1, TR2, RXRα, PPARα, and PPARγ were synthesized in a coupled transcription-translation rabbit reticulocyte lysate system (Promega). Recombinant HNF4, a gift from T. Gulick and D. Moore, was synthesized in HeLa cells infected with a recombinant vaccinia virus encoding HNF4; extract prepared from HeLa cells infected with wild-type (WT) vaccinia virus served as a control. Nuclear extracts of HeLa, HepG2, and Huh7 cells were prepared as described previously (10, 53). Polyclonal antisera against COUP-TF1 and HNF4 were prepared by injection of rabbits with *Escherichia coli*-synthesized glutathione *S*-transferase–COUP-TF1 and glutathione *S*-transferase–HNF4 fusion proteins, respectively.

Competition GMSAs were performed as described previously (52), with radiolabeled DR1 oligonucleotide as the probe, recombinant receptor proteins, and unlabeled double-stranded oligonucleotides used as competitors. ENHI is a 33-mer (5′-GATCACAGTACATGAACCTTTACCCCGTTGCTC-3′) containing the HRE in HBV enhancer I. ENHII is a 30-mer (5′-TACATAAGAGGA CTCTTGGACTCGCAGCAA-3′) containing the HNF4-binding site in enhancer II.

Cell-free transcription assays. Cell-free transcription reactions were performed with 25 to 100 ng of supercoiled plasmid DNA as template per 25-μl reaction as described previously (55). The relative quantities and locations of the 5′ ends of the resulting RNA products were determined by primer extension analysis as described previously with a 5′-end-labeled primer corresponding to HBV nt 1953 to 1931 (55). For the titration of nuclear receptors, double-stranded oligonucleotide DR1 or DR1$^-$ was incubated with the reaction mixture at 30°C for 30 min prior to the addition of the template DNA. The relative amounts of the pre-C and pregenomic RNAs were determined by quantitation of primer extension products resolved by polyacrylamide gel electrophoresis and detected with a PhosphorImager (Molecular Dynamics).

Transient transfection assays. Transient transfection of Huh7 and HepG2 cells was carried out by the calcium phosphate coprecipitation method as described previously (55). Each 100-mm-diameter dish of cells was transfected with a total of 25 μg of plasmid DNA. Plasmid pEQ176, encoding β-Gal, was included in each transfection mixture as an internal control (1.5 μg per 100-mm-diameter dish). Cells were incubated in a 1:1 mixture of F12 medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For cotransfection experiments with plasmids encoding RXRα and PPARγ, 10% charcoal-stripped fetal bovine serum (38) was used. Forty-eight hours posttransfection, nucleic acids were isolated from Huh7 cells with sodium dodecyl sulfate-EDTA (55) and from HepG2 cells by extraction with acid guanidinium thiocyanate-phenol-chloroform (8). Total cellular mRNA was purified by selection with oligo(dT). The relative quantities and locations of the 5′ ends of the mRNAs were also determined by primer extension as described above.

RESULTS

Identification of nuclear receptors that bind to the HBV DR1 HRE. To look for members of the nuclear receptor superfamily that bind the HBV DR1 HRE, GMSAs were performed with a synthetic, radiolabeled, 25-bp oligonucleotide corresponding to the sequences surrounding the HBV DR1 HRE as the probe (Fig. 1) and a variety of recombinant nuclear receptor proteins. As expected (55), recombinant HNF4 protein specifically bound to the DR1 oligonucleotide (Fig. 2A, lane 2). Approximately 80% of the HNF4-DNA complex was eliminated when a 30-fold molar excess of unlabeled DR1 oligonucleotide was included in the binding reaction but not when an equal amount of a variant of this oligonucleotide, mutant DR1$^-$, was included (Fig. 2A, lane 2 versus lanes 3 and 4). Moreover, the mobility of the HNF4 protein-DNA complex was altered by incubation with an HNF4-specific polyclonal antiserum (Fig. 2A, lane 8). These data confirm the sequence specificity of the binding of HNF4 to the DR1 HRE.

Similar experiments were performed with recombinant COUP-TF1 and a COUP-TF1-specific antiserum. As with HNF4, recombinant COUP-TF1 specifically bound the DR1 oligonucleotide (Fig. 2B, lanes 2 to 4), and the mobility of the protein-DNA complex shifted following incubation with the COUP-TF1-specific polyclonal antiserum (Fig. 2B, lane 7). Therefore, COUP-TF1 also binds the DR1 HRE in a sequence-specific manner.

Recombinant TR2 was also found to bind specifically to the DR1 oligonucleotide (Fig. 2C, lane 2). Whereas unlabeled DR1 oligonucleotide competed efficiently for binding, the DR1$^-$ oligonucleotide did not (Fig. 2C, lane 3 versus lane 4). A double-stranded oligonucleotide corresponding to the +55 site of the SV40 major late promoter, a region known to bind specifically to TR2 (30, 57), also efficiently competed with the radiolabeled DR1 probe for binding TR2 (Fig. 2C, lane 5).

PPARα and PPARγ are known to bind DR1s when heterodimerized with RXRs (34). As expected, we found that both PPARα and PPARγ, when present together with RXRα, bound the HBV DR1 HRE (Fig. 2D, lanes 5 and 8). On the other hand, neither PPARα, PPARγ, nor RXRα bound by itself (Fig. 2D, lanes 2 to 4). Thus, the observed protein-DNA complexes contained PPAR-RXR heterodimers. The amounts of the receptor-DNA complexes were reduced by approxi-

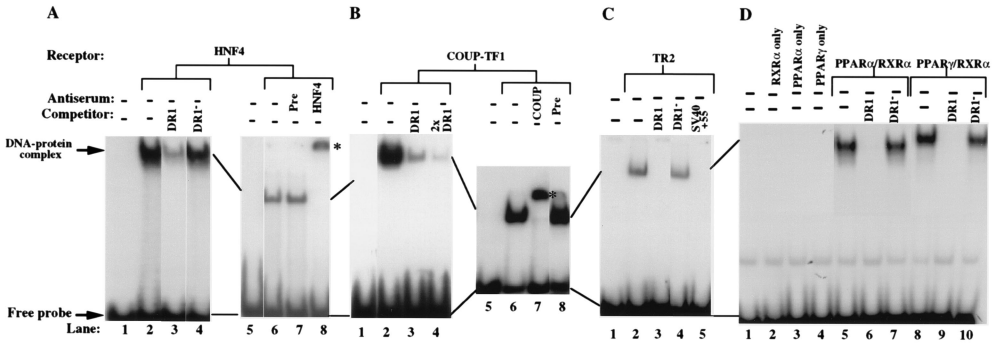

FIG. 2. Specific binding of nuclear receptors to the HBV DR1 HRE. (A) Autoradiogram of GMSA showing sequence-specific binding of recombinant HNF4 to the 25-bp double-stranded DR1 HRE oligonucleotide indicated in Fig. 1. A 30-fold molar excess of unlabeled DR1 or DR1$^-$ oligonucleotide was included as competitor in lane 3 or 4, respectively. Preimmune or HNF4-specific serum was included in lane 7 or 8, respectively. The position of the antibody-protein-DNA complex is indicated by an asterisk. (B) Autoradiogram of GMSA showing sequence-specific binding of recombinant COUP-TF1 to the HBV DR1 HRE. The experiment was performed as described for panel A except for the use of COUP-TF1 and a COUP-TF1-specific antiserum. Unlabeled DR1 oligonucleotide at 30- and 60-fold molar excesses was included as a competitors in lanes 3 and 4. The position of the antibody-protein-DNA complex is indicated by an asterisk. (C) Autoradiogram of GMSA showing sequence-specific binding of recombinant TR2. Included in lanes 3, 4, and 5 were 30-fold molar excesses of the competitor oligonucleotides DR1, DR1$^-$, and SV40 +55, respectively. The latter oligonucleotide contains the previously identified TR2-binding site present within the SV40 major late promoter. (D) Autoradiogram of GMSA showing that PPARα and PPARγ bind to DR1 oligonucleotide as heterodimers with RXRα (lanes 5 and 8, respectively) but not as monomers or homodimers (lanes 2 to 4). Included in lanes 6 and 9 and lanes 7 and 10 were 30-fold molar excesses of competitor oligonucleotides DR1 and DR1$^-$, respectively.

mately 90% by the addition of a 30-fold molar excess of unlabeled DR1 oligonucleotide but not of DR1$^-$ oligonucleotide (Fig. 2D, lane 6 versus lane 7 and lane 9 versus lane 10). Therefore, the HBV DR1 HRE can be bound specifically by PPARα-RXRα and PPARγ-RXRα heterodimers.

Competition GMSAs were conducted to compare the binding affinities of various nuclear receptors to oligonucleotides containing the DR1, ENHI, and ENHII HREs of HBV. As summarized in Table 1, the DR1 HRE is a strong binding site for the nuclear receptors studied here. The affinities for this site of COUP-TF1, the PPARs as heterodimers with the RXRs, HNF4, and TR2 are much higher than they are for the HRE in enhancer I. HNF4 was found to bind with similar high affinities to the ENHII and DR1 HREs. Thus, the binding of some of these receptors or related members of this superfamily to the DR1 HRE is likely of physiological relevance.

Several other recombinant nuclear receptor proteins, including human estrogen-related receptor α1 (24) and heterodimers of retinoic acid receptor α (33), human thyroid receptor α (29), and liver X receptor (54) with RXRα, were also tested by GMSAs for their binding to the DR1 oligonucleotide. No sequence-specific binding to the DR1 HRE was detected (56). In summary, we conclude that the HBV DR1 HRE can be bound sequence specifically and with high affinity by the nuclear receptors HNF4, COUP-TF1, TR2, PPARα-RXRα, and PPARγ-RXRα.

TABLE 1. Relative affinities of nuclear receptors for HBV HREs

Recombinant receptor protein	Relative binding affinity of indicated binding site[a]		
	ENHI HRE	ENHII HRE	DR1 HRE
COUP-TF1	+	−	+++
HNF4	+	+++	+++
TR2	+	−	++
PPARα-RXRα	+	−	++

[a] Determined by competition GMSAs as described in Materials and Methods. −, no binding detected; +, ++, and +++, binding with low, moderate, and high affinity, respectively.

Binding to the HBV DR1 HRE of HNF4 and COUP-TF1 present in hepatoma cells. HBV normally replicates in the liver, an organ known to be abundant for HNF4 (45) and to contain COUP-TF1 (37). It also replicates in hepatoma cell lines (44). To test for the presence of these nuclear receptors in the human hepatoma cell lines HepG2 and Huh7, nuclear extracts were prepared from them and the nonliver cell line HeLa as a control. DNA-protein complexes were observed in GMSAs performed with these extracts and radiolabeled DR1 probe (Fig. 3). Most of these complexes could be competed away with unlabeled WT but not mutant DR1 oligonucleotide (Fig. 3A, lanes 3 versus 4 and 9 versus 10; Fig. 3B, lane 3 versus

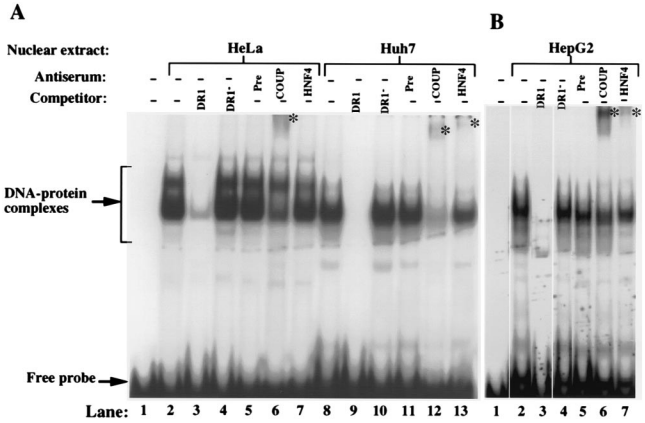

FIG. 3. Specific binding of nuclear receptors in nonliver and hepatoma cells to the HBV DR1 HRE. (A) Autoradiogram showing GMSA with HeLa and Huh7 nuclear extracts and radiolabeled DR1 oligonucleotide. For competition GMSAs, a 30-fold molar excess of unlabeled DR1 or DR1$^-$ oligonucleotide was included as the competitor in lanes 3, 4, 9, and 10 as indicated. For immunoshift assays, preimmune, COUP-TF1-specific or HNF4-specific serum was included in lanes 5 to 7 and 11 to 13 as indicated. The positions of the antibody-protein-DNA complexes are indicated by asterisks to the immediate right in lanes 6, 12, and 13. (B) Autoradiogram showing GMSA with HepG2 nuclear extract and DR1 oligonucleotide. Competition GMSAs and immunoshift assays were performed as described above. The positions of the antibody-protein-DNA complexes are indicated by asterisks adjacent to lanes 6 and 7.

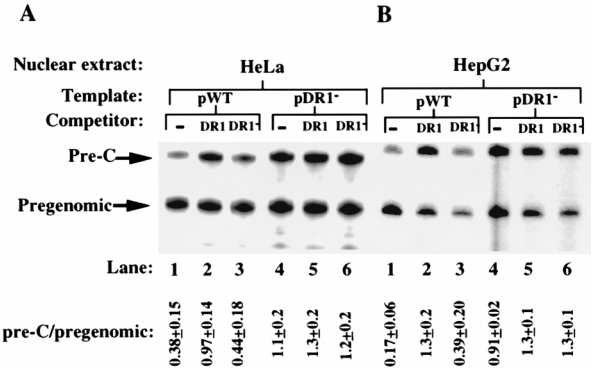

FIG. 4. Repression of pre-C RNA synthesis in cell-free transcription systems directed by DR1 HRE-binding factor(s). Each reaction mixture contained 25 ng of supercoiled pWT or pDR1$^-$ DNA and 8 μl of nuclear extract from HeLa (A) or HepG2 (B) cells. A 2,000-fold molar excess of unlabeled, double-stranded DR1 or DR1$^-$ oligonucleotide was included as the competitor where indicated. The 5′ ends of the resulting RNAs were determined by primer extension analysis. Shown is an autoradiogram of the cDNA products electrophoresed in a 7 M urea–8% polyacrylamide gel. The numbers at the bottom of this and subsequent figures indicate the molar ratios of pre-C/pregenomic RNA present in each lane; they were determined with a PhosphoImager and represent the means ± standard error of the means from three experiments similar to the one shown in each figure.

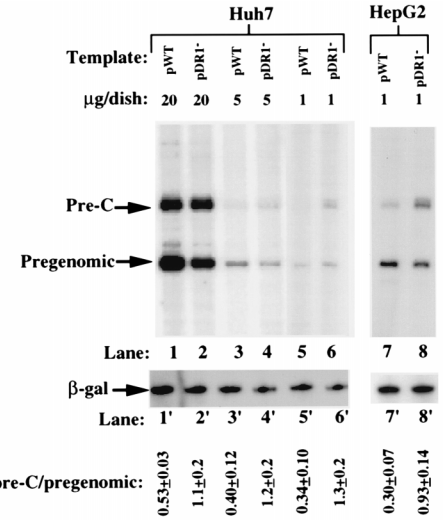

FIG. 5. Synthesis of pre-C RNA is repressed in transiently transfected hepatoma cells at low template concentrations. Huh7 (A) and HepG2 (B) cells were transiently transfected with the indicated amounts of the indicated plasmid DNAs. Forty-eight hours later, whole-cell mRNA was isolated. The 5′ ends of the pre-C and pregenomic RNAs were determined by primer extension, followed by electrophoresis in a 7 M urea–8% polyacrylamide gel. One-third (A, lanes 1 to 4) or two-thirds (A, lanes 5 and 6; B, lanes 1 and 2) of the RNA from a 100-mm-diameter dish of cells was used in each primer extension reaction. Shown are the resulting autoradiograms. Concurrent analysis of β-Gal synthesized from the cotransfected plasmid pEQ176 served as an internal control.

4). Thus, these complexes are probably specific to the DR1 HRE.

Two or three major complexes were observed with the HeLa nuclear extract (Fig. 3A, lane 2). Approximately 60% of the predominant DNA-protein complex formed on the DR1 probe was eliminated or altered in mobility by incubation with a COUP-TF1-specific antiserum (Fig. 3A, lane 6). Therefore, one of the major proteins present in HeLa cells that can bind the DR1 HRE is COUP-TF1. On the other hand, incubation with an HNF4-specific antiserum had little, if any, effect (Fig. 3A, lane 7). We also failed to detect HNF4 protein in HeLa cell nuclear extract by immunoblotting (56). Therefore, as expected, HeLa cells are abundant in the ubiquitous receptor COUP-TF1 but deficient in the liver-enriched receptor HNF4.

Immunoshift assays were also performed with nuclear extract prepared from Huh7 cells. Approximately 80% of one of the bands in the GMSA was eliminated or altered in mobility by incubation with the COUP-TF1-specific antiserum, indicating that it contained COUP-TF1 (Fig. 3A, lane 12). The HNF4-specific antiserum immunoshifted approximately 20% of the protein-DNA complex from the major band (Fig. 3A, lane 13). Similar results were obtained with the HepG2 cell nuclear extract (Fig. 3B). The protein-DNA complexes not eliminated or altered in mobility by the COUP-TF1- and HNF4-specific antisera probably included ones containing other nuclear receptors such as the PPARs and RXRs (40). Therefore, we conclude, as expected, that the nuclear receptors COUP-TF1 and HNF4 are present in these hepatoma cell lines and can specifically bind to the HBV DR1 HRE.

Repression of pre-C RNA synthesis. The effects on RNA synthesis of the binding of nuclear receptors to the HBV DR1 HRE were examined with cell-free transcription systems derived from nuclear extracts of HeLa and HepG2 cells. Whereas synthesis of pregenomic RNAs was not significantly affected by mutation of the DR1 HRE, synthesis of the pre-C RNAs reproducibly increased three- to fivefold (Fig. 4A and B, lane 1 versus lane 4). Therefore, binding of nuclear receptors present in these nuclear extracts to the DR1 HRE probably represses transcription specifically from the pre-C promoter.

Since the DR1 HRE spans the TATA box-like sequence of the pre-C promoter (55) (Fig. 1), an alternative hypothesis consistent with these data is that the sequence alterations in the DR1$^-$ mutant change the affinity of this basal element of this promoter for a general transcription factor (e.g., TFIID). To distinguish between these two hypotheses, we also performed cell-free transcription assays in the presence of a 2,000-fold molar excess of oligonucleotide DR1 or DR1$^-$. If the difference in synthesis of the pre-C RNAs is due to binding of nuclear receptors to the DR1 HRE, the presence of the WT DR1 oligonucleotide would be expected to titrate them out of the nuclear extracts, leading to derepression of the pre-C promoter. On the other hand, the DR1$^-$ oligonucleotide, unable to bind the nuclear receptors, would fail to do so. This is what was found (Fig. 4A and B, lane 1 versus lanes 2 and 3). Addition of either oligonucleotide had no effect on synthesis of the pre-C RNAs when plasmid pDR1$^-$ was used as the template since the nuclear receptors were already unable to bind the promoter efficiently (Fig. 4A and B, lanes 4 to 6). Therefore, we conclude that repression of synthesis of pre-C RNA in the cell-free transcription systems is due to binding of nuclear receptors to the HBV DR1 HRE. However, since these extracts contain multiple nuclear receptors (Fig. 3), it is unclear which ones are physiologically important in mediating this observed effect.

To confirm the validity of this finding, similar experiments were performed by transient transfection of various amounts of WT and DR1 mutant plasmid DNA into the hepatoma cell lines Huh7 and HepG2. When the plasmid DNAs were transfected into either cell line at a low concentration (e.g., 1 μg of plasmid DNA per 100-mm-diameter dish), the amount of the pre-C RNA accumulated in the cells by 48 h posttransfection with pWT was one-third to one-fourth of the amount accumulated with pDR1$^-$ (Fig. 5, lane 5 versus lane 6 and lane 7 versus lane 8). However, when the plasmid DNA concentration was

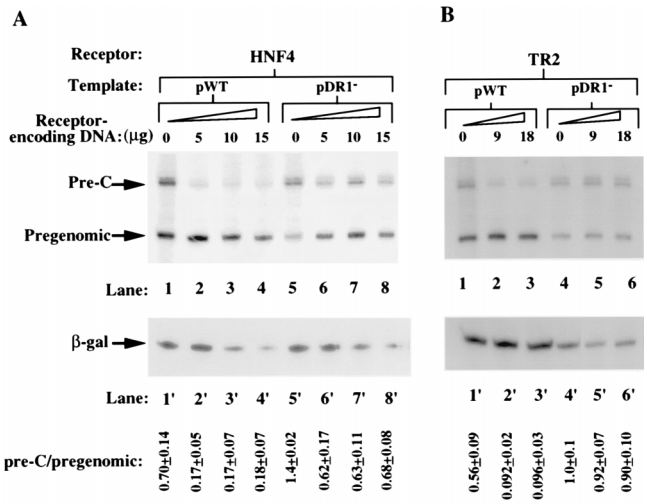

FIG. 6. HNF4 and TR2 specifically repress synthesis of pre-C RNA in Huh7 cells. (A) Huh7 cells were cotransfected (5 μg per 100-mm-diameter dish) with pWT (lanes 1 to 4) or pDR1$^-$ (lanes 5 to 8) as the template, the parental vector plasmid pCDM8 (lanes 1 and 5), and the indicated amount of the HNF4-encoding plasmid pCDMHNF4. Subsequently, the cells were incubated and their mRNAs were harvested and analyzed as described for Fig. 5. One-third of a 100-mm-diameter dish of cells was used in each primer extension reaction. (B) Huh7 cells were cotransfected with the template plasmid pWT (5 μg; lanes 1 to 3) or pDR1$^-$ (5 μg; lanes 4 to 6), the parental vector plasmid pSG5 (9 μg; lanes 1 and 4), and the indicated amounts of the TR2-encoding plasmid pSG5TR2 and analyzed as described for panel A.

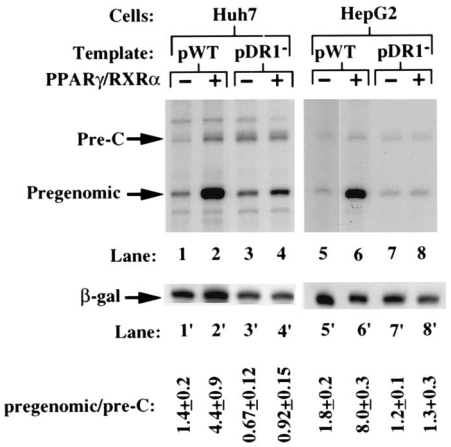

FIG. 7. PPARγ-RXRα specifically activates synthesis of pregenomic RNA in Huh7 and HepG2 cells. Cells were cotransfected with 6 μg per 100-mm-diameter dish of pWT (lanes 1, 2, 5, and 6) or pDR1$^-$ (lanes 3, 4, 7, and 8) as template DNA plus 2 μg of pSV-SPORT-PPARγ2 and 0.5 μg of pSGhRXRα (lanes 2, 4, 6, and 8) or an equal amount of the parental plasmids (lanes 1, 3, 5, and 7). The isolation and analysis of mRNAs were performed as described for Fig. 5. One-third of a 100-mm-diameter dish of cells was used in each primer extension reaction.

higher (e.g., 20 μg of plasmid DNA per 100-mm-diameter dish), the effect of the mutation on accumulation of the pre-C RNA was significantly diminished (Fig. 5, lanes 1 and 2). Therefore, we conclude that sequence-specific repression of the pre-C promoter by nuclear receptors can also be observed in transiently transfected hepatoma cells when the template copy number is low—i.e., under conditions in which most DR1 HRE sites are likely bound by nuclear receptors.

Selective repression of pre-C RNA synthesis by HNF4 and TR2. To investigate the effects of individual members of the nuclear receptor superfamily on synthesis of the pre-C and pregenomic RNAs, Huh7 cells were cotransfected with template DNA and various concentrations of plasmids encoding nuclear receptors. The presence of the HNF4-encoding plasmid inhibited synthesis of the pre-C RNA approximately fourfold and slightly activated synthesis of the pregenomic RNA (Fig. 6A, lanes 1 to 4). On the other hand, overexpression of HNF4 had only a twofold effect on synthesis of the pre-C and pregenomic RNAs when plasmid pDR1$^-$ was used as the template DNA (Fig. 6A, lanes 5 to 8). The twofold change observed in the pre-C/pregenomic RNA ratio with pDR1$^-$ when HNF4 was overexpressed was likely due to the binding of HNF4 to the DR1 HRE being reduced but not completely eliminated by the DR1$^-$ mutations (56) or the binding of HNF4 to the ENHII HRE resulting in activation of synthesis of pregenomic RNA (18). A fivefold reduction in synthesis of the pre-C RNA was observed when the TR2-encoding plasmid pSG5-TR2 was cotransfected with the WT but not the mutant template DNA (Fig. 6B, lanes 1 to 3 versus lanes 4 to 6, respectively). Therefore, we conclude that overexpression of the nuclear receptors HNF4 and TR2 in Huh7 cells can selectively repress synthesis of the pre-C RNAs via binding of nuclear receptors to the HBV DR1 HRE.

Selective activation of pregenomic RNA synthesis by PPARγ-RXRα. A similar cotransfection experiment was performed to investigate the effects of PPARγ-RXRα on synthesis of the pre-C and pregenomic RNAs in Huh7 and HepG2 cells. In this case, the expression of PPARγ plus RXRα led to increased synthesis of the pregenomic RNA by approximately three-fold in Huh7 cells (Fig. 7, lane 1 versus lane 2) and four- to five-fold in HepG2 cells (Fig. 7, lane 5 versus lane 6). On the other hand, overexpression of PPARγ-RXRα had little, if any, effect on synthesis of pre-C RNA. When plasmid pDR1$^-$ was used as the template in the cotransfection experiments, no significant activation of synthesis of pregenomic RNA was observed (Fig. 7, lane 4 versus lane 3 and lane 8 versus lane 7). Therefore, we conclude that expression of PPARγ-RXRα can selectively activate synthesis of the pregenomic RNA via binding of the heterodimer to the HBV DR1 HRE.

Repression of both pre-C and pregenomic RNA synthesis by COUP-TF1. A similar cotransfection experiment was also performed with a COUP-TF1-encoding plasmid. In this case, overexpression of the nuclear receptor resulted in a 5- to 10-fold reduction in synthesis of both the pre-C and pregenomic RNAs from the WT template (Fig. 8A, lanes 1 to 4). This COUP-TF1-mediated repression required binding to the DR1 HRE since no significant repression of RNA synthesis from either promoter was observed when pDR1$^-$ was used as the template (Fig. 8A, lanes 5 to 8). Therefore, as opposed to HNF4, TR2, and PPAR-RXR, the binding of COUP-TF1 to the DR1 HRE appears to coordinately affect transcription from the pre-C and pregenomic promoters.

To confirm that the binding of COUP-TF1 is not simply interfering with transcription initiated at these overlapping promoters, we repeated this experiment with the template pMinser, a variant of pWT containing a 15-bp insertion between the initiators of these two promoters. Synthesis of both the pre-C and pregenomic RNAs was still repressed five- to seven-fold by overexpression of COUP-TF1 (Fig. 8B, lanes 3 to 6). Therefore, we conclude that overexpression of COUP-TF1 in Huh7 cells coordinately represses synthesis of both the pre-C and pregenomic RNAs via binding the DR1 HRE even when the basal elements of the pregenomic promoter are not overlapping those of the pre-C promoter.

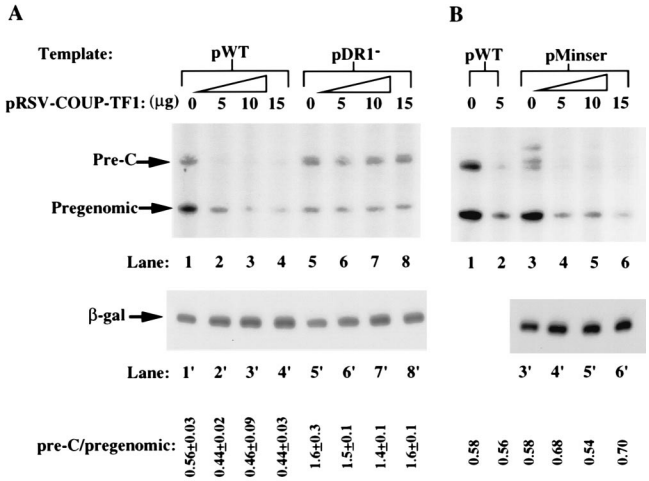

FIG. 8. Overexpression of COUP-TF1 leads to specific repression of synthesis of both the pre-C and pregenomic RNAs in Huh7 cells. (A) Huh7 cells were cotransfected with pWT (5 μg; lanes 1 to 4) or pDR1$^-$ (5 μg; lanes 5 to 8) as the template and the indicated amounts of pRSV-COUP-TF1 (lanes 2 to 4 and 6 to 8) or its parental vector pRSV0 (5 μg; lanes 1 and 5). The cells were incubated and their mRNAs were analyzed as described for Fig. 6. (B) Huh7 cells were cotransfected with pWT (5 μg; lanes 1 and 2) or pMinser (5 μg; lanes 3 to 6) and the indicated amounts of pRSV-COUP-TF1 (lanes 2 and 4 to 6) or its parental vector pRSV0 (5 μg; lanes 1 and 3).

Modulation by nuclear receptors in a cell-free transcription system. To confirm the validity of our finding that the binding of nuclear receptors to the DR1 HRE can affect transcription from the pre-C and pregenomic promoters, we also performed cell-free transcription assays in the presence of exogenous recombinant nuclear receptor proteins. As was observed with the transfected cells, addition of recombinant COUP-TF1 to a HeLa cell-derived cell-free transcription system resulted in a coordinate 8- to 10-fold reduction in synthesis of both the pre-C and pregenomic RNAs from the WT but not from the DR1 HRE mutant template (Fig. 9, lanes 1 to 5). On the other hand, addition of recombinant HNF4 to the cell-free transcription system reduced synthesis primarily of the pre-C RNA (Fig. 9, lanes 6 to 8). These findings agree well with the ones obtained with the transfected cells. In summary, we conclude that overexpression of different nuclear receptors can exert distinct effects on transcription from the pre-C and pregenomic promoters via binding to the DR1 HRE.

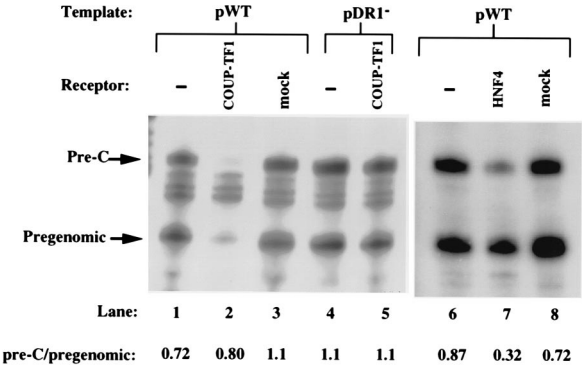

FIG. 9. Effects of recombinant COUP-TF1 and HNF4 on synthesis of pre-C and pregenomic RNAs in a cell-free transcription system. Each reaction mixture of 25 μl contained 100 ng of the indicated DNA as template, 8 μl of HeLa cell nuclear extract, and recombinant COUP-TF1 (lanes 2 and 5), unprogrammed reticulocyte lysate (lane 3), recombinant HNF4 (lane 7), or lysate from WT vaccinia virus-infected HeLa cells (lane 8). After incubation, the samples were processed by phenol extraction and ethanol precipitation prior to primer extension analysis and polyacrylamide gel electrophoresis. The numbers at the bottom are the pre-C/pregenomic RNA ratios from this experiment.

DISCUSSION

We have shown here that a DR1 HRE present within the pre-C and pregenomic promoters of HBV can be bound sequence specifically by several members of the nuclear receptor superfamily including COUP-TF1, HNF4, TR2, PPARα-RXRα, and PPARγ-RXRα (Fig. 2). COUP-TF1 and HNF4, as present in nuclear extracts made from hepatoma cells, were also shown to bind the DR1 HRE (Fig. 3). These findings suggest that these interactions likely can take place during infection of liver cells by HBV. Analysis in both a cell-free transcription system (Fig. 4) and transiently transfected cells (Fig. 5) indicated that transcription from the pre-C promoter of HBV can be repressed by the sequence-specific binding of some members of the nuclear receptor superfamily to the DR1 HRE. This repression can be relieved by titration with competitor DNA (Fig. 4) or high template copy number (Fig. 5). Overexpression of specific nuclear receptors in transfected Huh7 cells showed that whereas some receptors can specifically repress synthesis of pre-C RNA (e.g., HNF4 and TR2) (Fig. 6) or activate synthesis of pregenomic RNA (e.g., PPARγ-RXRα) (Fig. 7), others (e.g., COUP-TF1) can coordinately repress synthesis of both the pre-C and pregenomic RNAs (Fig. 8). These effects were dependent on the integrity of the DR1 HRE (Fig. 6 to 8). Similar results were observed with the addition of recombinant receptor protein to a cell-free transcription system (Fig. 9). Therefore, we conclude that members of the nuclear receptor superfamily can differentially regulate transcription from the pre-C and pregenomic promoters via interactions with the DR1 HRE.

Recently, Raney et al. (40) also reported their independent identification of this DR1 HRE, its binding by HNF4 and PPARα-RXRα, and the effects of these receptors on transcription from the pre-C/pregenomic promoter region of HBV. Their binding data are in excellent agreement with the data reported here. Unfortunately, the two sets of transcription data cannot be compared directly: (i) their use of a luciferase reporter did not enable them to distinguish between synthesis of pre-C and pregenomic RNAs, and (ii) they performed their experiments with the dedifferentiated hepatoma cell line HepG2.1, which is distinct from the well-differentiated hepatoma cell lines HepG2 and Huh7 used in the studies reported here (39).

DR1 HRE is highly conserved in the HBV genome. We examined the sequences of the 29 independent human HBV isolates currently present in the DNA database of the Genetics Computer Group (Madison, Wis.). Nineteen of these isolates contain the DR1 HRE sequence 5′-AGGTTAAAGGTCT-3′. These 19 belong to the HBV subtypes adr, ayw, adw, and ayr. Seven of the remaining known HBV isolates contain only one clear half-site sequence, 5′-AGGTTA-3′ or 5′-AGGTCT-3′; the other potential half-site contains two or three differences from the consensus half-site sequence 5′-AGGTCA-3′. These "mutated" DR1 HREs may still allow for weak binding by nuclear receptors. In only 3 of the 29 isolates was the sequence variation different to the point that nuclear receptors are not likely to bind to them. Thus, it appears that the presence of the DR1 HRE is highly conserved in nature.

An alternative explanation for why the sequence in this region of the HBV genome is highly constrained is that it also functions to encode the X protein (Fig. 1). It has been reported

that two regions of the amino acid sequence of the X protein are highly conserved, and amino acid substitutions due to variations in the DR1 HRE sequence reduce dramatically the activity of the X protein as a transactivator (41). The DR1 HRE also spans the TATA box-like sequence 5′-TTAAA-3′ of the pre-C promoter (Fig. 1) (55), thus placing further constraint on this sequence. Nevertheless, even the three GC base pairs within the DR1 HRE at nt 1759, 1765, and 1768 in which alterations would not affect coding of the X protein or the TATA box-like sequence of the pre-C promoter remain highly conserved, with variations existing at one or more of these positions in only seven of the isolates. Therefore, we conclude that the DR1 HRE site is probably highly conserved in the human HBV genome because the binding of nuclear receptors is important for the life cycle of the virus (see also below).

DR1 HRE is a strong binding site for nuclear receptors abundantly present in liver cells. We (Fig. 2) and others (40) have identified several nuclear receptors that can specifically bind DNA containing the DR1 HRE. Among these nuclear receptors, COUP-TF1 is an orphan member that plays roles in regulating embryonic development (37, 49). Immunoshift assays showed that COUP-TF1 is abundantly present in the hepatoma cell lines Huh7 and HepG2 as well as in HeLa cells (Fig. 3). Competition GMSAs showed that the affinity of COUP-TF1 for the DR1 HRE is at least 10-fold higher than it is for the previously identified HRE present in the enhancer I region of the HBV genome (Table 1) (56). Transcription studies showed that COUP-TF1 binding to this site coordinately regulates both promoters (Fig. 8 and 9). However, given our finding that the DR1 HRE is involved in differential, not coordinate, regulation of the pre-C and pregenomic promoters (Fig. 4 and 5), the physiological significance, if any, of the binding of COUP-TF1 to the DR1 HRE remains unclear.

HNF4 is a liver-enriched member of the nuclear receptor superfamily whose ligand also remains unknown (45). It has been shown to function as a transactivator of several liver-specific promoters (5, 13, 19, 25, 27). Competition GMSAs showed that the affinity of HNF4 for the DR1 HRE was similar to its affinity for the previously reported HNF4-binding site in enhancer II (18) and fivefold higher than its affinity for the HRE in enhancer I (Table 1) (56). Although less abundant than COUP-TF1 (Fig. 3), HNF4 is one member of the nuclear receptor superfamily whose activities are consistent with its playing physiological roles in regulation of HBV gene expression via the DR1 HRE (Fig. 4 and 5 versus Fig. 6A and 9).

TR2, another orphan member of the nuclear receptor superfamily, is present in liver cells at approximately one-third of its level in human testis (7). With only one other TR2-binding site known to date (30, 57), we have not pursued further the relevance of the binding of TR2 to the DR1 HRE beyond noting its sequence-specific repression of pre-C RNA synthesis (Fig. 6B).

We also showed here that PPARα and PPARγ as heterodimers with RXRα, but not by themselves, can also bind the DR1 HRE with high affinity (Fig. 2D; Table 1). The PPARs are members of the nuclear receptor superfamily that play important roles in adipogenesis and lipid metabolism (2, 23). The effects on transcription of the binding of the receptors to the DR1 HRE (Fig. 7) are also consistent with these receptors playing physiological roles in regulating expression of HBV.

Which of these multiple nuclear receptors are actually of physiological importance in the regulation of HBV gene expression via the DR1 HRE remains unknown. We speculate that several members of this huge superfamily, including ones yet to be identified, play roles by competing for binding to the DR1 HRE. Which receptors bind and what effects they have are likely dependent on numerous factors, including the physiological state of the cell, the amount of HBV DNA present in the cell and the presence or absence of the ligands to these various relevant receptors.

Regulation of the pre-C promoter by nuclear receptors. When either the DR1 HRE was mutated (Fig. 4, 6, 7, and 8) or the template copy number was high (Fig. 5), the pre-C and pregenomic RNAs were synthesized at similar levels. On the other hand, pre-C RNA synthesis was repressed while pregenomic RNA synthesis was unaffected or activated when transcription occurred from the WT template at low copy number with the nuclear receptors present at their normal physiological concentrations either in transiently transfected cells (Fig. 5) or in the cell-free assay systems used here (Fig. 4). Likewise, Farza et al. (11) have reported that pre-C and pregenomic RNA levels are similar in most organs of HBV transgenic mice except the liver, where the amount of pre-C RNA is low. The pre-C RNA level is also low in the liver of HBV-infected chimpanzees (11). Thus, HNF4 and other endogenous HNF4-like receptors present in limited amounts are likely the physiologically relevant players involved in regulation of the pre-C and pregenomic promoters via this DR1 HRE rather than abundant nuclear receptors such as COUP-TF1 (Fig. 3).

We directly demonstrated the involvement of HNF4 in repression of transcription from the pre-C promoter by both overexpression (Fig. 6A) and addition (Fig. 9) of HNF4 protein. Since this DR1 HRE site spans the TATA box-like sequence of this promoter, the mechanism of repression may involve passive interference with the formation of functional preinitiation complexes. However, the binding of PPARγ-RXRα to this site activates transcription from the pregenomic promoter without affecting transcription from the pre-C promoter (Fig. 7). Thus, the mechanism of repression by HNF4 is probably an active one, e.g., one involving competition with upstream-binding coactivators for binding to general transcription factors.

Regulation of the pregenomic promoter by nuclear receptors. The binding of PPARγ-RXRα to the DR1 HRE leads to differential activation of pregenomic RNA synthesis. Overexpression of PPARγ and RXRα in transiently transfected Huh7 and HepG2 cells led to a three- to fivefold increase of synthesis of pregenomic RNA (Fig. 7). Similar results were observed when only PPARγ was overexpressed but not when only RXRα was overexpressed (56). This finding suggests that the abundance of RXRα is likely high, while that of PPARγ is likely relatively low in the hepatoma cells used in this study. Consistent with this suggestion is the fact that the level of RXRα mRNA is much higher than that of PPARγ mRNA in mouse liver (47).

The natural ligand of PPARγ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), has been recently identified and shown to play a role in the activation of transcription from promoters containing PPAR HREs (12, 26). The level of its precursor, Δ^{12}-PGJ_2, is 2- to 10-fold higher in males than in females (20). Possibly, this gender difference in the level of PGJ_2 contributes to the male's higher incidence of persistent HBV infection, liver cirrhosis, and HCC (1, 46). Thus, it will be of considerable interest to determine the effects of 15d-PGJ_2 and other ligands of PPARγ on the synthesis of the pre-C and pregenomic RNAs.

As opposed to HNF4 and PPARγ-RXRα, the binding of COUP-TF1 was found to repress transcription from the pregenomic promoter (Fig. 8A; Fig. 9). Our finding that repression still occurred in the 15-bp insertion mutant pMinser (Fig. 8B) suggests that repression of the pregenomic promoter by COUP-TF1 is an active process (32). In further support of this

hypothesis is our finding that COUP-TF1 fails to repress either pre-C or pregenomic RNA synthesis in transiently transfected Huh7 cells when the upstream regulatory region (nt 1403 to 1729) is deleted (56).

Thus, we conclude that various liver-enriched members of the nuclear receptor superfamily can differentially regulate the pre-C and pregenomic promoters of HBV under the experimental assay conditions used here. However, it remains to be determined which receptors and ligands are truly the ones responsible for regulation of these HBV promoters under physiological conditions in vivo.

Effects of receptor binding on the life cycle of HBV. Pre-C RNA encodes precore protein which is proteolytically cleaved to yield e antigen. Thus, repression of pre-C RNA synthesis should result in reduced synthesis of both the precore and e proteins. The precore protein has been shown to inhibit HBV replication both in the liver of transgenic mice (17) and in transiently transfected hepatoma cells (28) by exerting a dominant negative effect, probably at the level of nucleocapsid particle maturation or stability. Thus, we speculate that repression of synthesis of pre-C RNA could be advantageous to the virus early in infection because a low level of precore protein in liver cells would enable viral DNA synthesis to occur efficiently. HBV DNA synthesized in the nucleocapsids could then enter the nucleus, thereby increasing the copy number of covalently closed circular DNA that can function as template for further RNA synthesis. Once the template copy number in the nucleus is sufficiently high, repression of the pre-C promoter might diminish due to partial titration of nuclear receptors, thereby leading to more efficient synthesis of pre-C RNA and precore protein and regulated production of mature virion particles. Low-level synthesis of e antigen early in infection might also be important to the viral life cycle. In this case, it might delay the onset of the host immune response against the viral infection until after the infection has become established.

Synthesis of the pregenomic RNA is also a pivotal step in the replicative cycle of HBV because it both encodes the proteins C and P, which are essential for the formation of nucleocapsids, and serves as the template for viral DNA synthesis. Thus, the pregenomic and pre-C RNAs exert opposite effects on viral replication. Therefore, to maintain different levels of viral replication in acute and chronic HBV infections and in asymptomatic HBV carriers, the virus must have mechanisms for the temporal and differential regulation of synthesis of the pre-C and pregenomic RNAs from these overlapping promoters. The data presented here suggest that this regulation can be achieved, at least in part, through interactions of various nuclear receptors with the DR1 HRE.

HBV variants with mutations in the DR1 HRE have been isolated from patients with chronic and fulminant hepatitis B (36, 42). These mutations, which alter both the DR1 HRE and the TATA box-like sequence of the pre-C promoter, mainly affect synthesis of the pre-C RNA. Recently, Buckwold et al. (3) analyzed the effects of the mutations in one of these naturally occurring variants (A to T at nt 1764; G to A at nt 1766) on the synthesis of the viral RNAs and viral replication in transiently transfected hepatoma cells. They found that this double mutation significantly reduced synthesis of pre-C RNA, but not pregenomic RNA, leading to increased production of virion particles. Combining their findings with our own, we predict that the binding of nuclear receptors such as HNF4 to the WT DR1 HRE would, likewise, result in more efficient virion production with low e antigen synthesis.

In summary, we conclude that various nuclear receptors naturally abundant in liver cells can differentially modulate synthesis of the pre-C and pregenomic RNAs through binding to the DR1 HRE site present in the pre-C/pregenomic promoter region. We speculate that the natural ligands to nuclear receptors and analogs of them may be useful as therapeutic agents in the treatment of HBV infections. For example, one ligand which represses synthesis of the pregenomic RNA may be useful in the treatment of fulminant hepatitis B, while another ligand which activates synthesis of pre-C RNA may be helpful in curtailing HBV virion production and eliciting an earlier immune response in acutely infected patients.

ACKNOWLEDGMENTS

We thank Tod Gulick and David Moore for recombinant HNF4 protein and plasmid pT7-PPARα, M.-J. Tsai for plasmids pCOUP-TF1 and pRSV-COUP-TF1, P. Chambon for plasmid pSGhRXRα, C. Chang for plasmid pSG5-TR2, and M. Spiegelman for plasmid pSV-SPORT-PPARγ2. We also thank Paul Lambert, Dan Loeb, and members of our laboratory for helpful discussions and comments on the manuscript and Dana Willard for assistance with the DNA database search.

This work was supported by Public Health Service research grants CA07175 and CA22443 from the National Institutes of Health.

REFERENCES

1. **Beasley, R. P.** 1988. Hepatitis B virus: the major etiology of hepatocellular carcinoma. Cancer. **61:**1942–1956.
2. **Brun, R. P., P. Tontonoz, B. M. Forman, R. Ellis, J. Chen, R. M. Evans, and B. M. Spiegelman.** 1996. Differential activation of adipogenesis by multiple PPAR isoforms. Genes Dev. **10:**974–984.
3. **Buckwold, V. E., Z. Xu, M. Chen, T. S. B. Yen, and J.-H. Ou.** 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J. Virol. **70:**5845–5851.
4. **Butz, K., and F. Hoppe-Seyler.** 1993. Transcriptional control of human papillomavirus (HPV) oncogene expression: composition of the HPV type 18 upstream regulatory region. J. Virol. **67:**6476–6486.
5. **Carter, M. E., T. Gulick, B. D. Raisher, T. Caira, J. A. A. Ladias, D. D. Moore, and D. P. Kelly.** 1993. Hepatocyte nuclear factor-4 activates medium chain acyl-CoA dehydrogenase gene transcription by interacting with a complex regulatory element. J. Biol. Chem. **268:**13805–13810.
6. **Castelein, H., T. Gulick, P. E. Declercq, G. P. Mannaerts, D. D. Moore, and M. I. Baes.** 1994. The peroxisome proliferator activated receptor regulates malic enzyme gene expression. J. Biol. Chem. **269:**26754–26758.
7. **Chang, C., and J. Kokontis.** 1988. Identification of a new member of the steroid receptor super-family by cloning and sequence analysis. Biochem. Biophys. Res. Commun. **155:**971–977.
8. **Chomczynski, P.** 1994. Current protocols in molecular biology, p. 4.2.4.–4.2.8. John Wiley & Sons, Inc, New York, N.Y.
9. **Cooney, A. J., S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai.** 1992. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. Mol. Cell. Biol. **12:**4153–4163.
10. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
11. **Farza, H., M. Hadchouel, J. Scotto, P. Tiollais, C. Babinet, and C. Pourcel.** 1988. Replication and gene expression of hepatitis B virus in a transgenic mouse that contains the complete viral genome. J. Virol. **62:**4144–4152.
12. **Forman, B. M., P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman, and R. M. Evans.** 1995. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell **83:**803–812.
13. **Galson, D. L., T. Tsuchiya, D. S. Tendler, L. E. Huang, Y. Ren, T. Ogura, and H. F. Bunn.** 1995. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol. Cell. Biol. **15:**2135–2144.
14. **Ganem, D.** 1996. Hepadnaviridae and their replication, p. 2703–2739. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
15. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56:**651–693.
16. **Garcia, A. D., P. Ostapchuk, and P. Hearing.** 1993. Functional interaction of nuclear factors EF-C, HNF-4, and RXRα with hepatitis B virus enhancer I. J. Virol. **67:**3940–3950.
17. **Guidotti, L. G., B. Matzke, C. Pasquinelli, J. M. Shoenberger, C. E. Rogler, and F. V. Chisari.** 1996. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. J. Virol. **70:**7056–7061.
18. **Guo, W., M. Chen, T. S. B. Yen, and J.-H. Ou.** 1993. Hepatocyte-specific

expression of the hepatitis B virus core promoter depends on both positive and negative regulation. Mol. Cell. Biol. **13:**443–448.
19. **Hall, R. K., F. M. Sladek, and D. K. Granner.** 1995. The orphan receptors COUP-TF and HNF-4 serve as accessory factors required for induction of phospho*enol*pyruvate carboxykinase gene transcription by glucocorticoids. Proc. Natl. Acad. Sci. USA **92:**412–416.
20. **Hirata, Y., H. Hayashi, S. Ito, Y. Kikawa, M. Ishibashi, M. Sudo, H. Miyazaki, M. Fukushima, S. Narumiya, and O. Hayaishi.** 1988. Occurrence of 9-deoxy-Δ^9,Δ^{12}-13,14-dihydroprostaglandin D_2 in human urine. J. Biol. Chem. **263:**16619–16625.
21. **Huan, B., and A. Siddiqui.** 1992. Retinoid X receptor RXRα binds to and trans-activates the hepatitis B virus enhancer. Proc. Natl. Acad. Sci. USA **89:**9059–9063.
22. **Huan, B., M. J. Kosovsky, and A. Siddiqui.** 1995. Retinoid X receptor α transactivates the hepatitis B virus enhancer 1 element by forming a heterodimeric complex with the peroxisome proliferator-activated receptor. J. Virol. **69:**547–551.
23. **Issemann, I., and S. Green.** 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature **347:**645–650.
24. **Johnston, S. D., X. Liu, F. Zuo, T. L. Eisenbraun, S. R. Wiley, R. J. Kraus, and J. E. Mertz.** 1997. Estrogen-related receptor α1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. Mol. Endocrinol. **11:**342–352.
25. **Kimura, A., A. Nishiyori, T. Murakami, T. Tsukamoto, S. Hata, T. Osumi, R. Okamura, M. Mori, and M. Takiguchi.** 1993. Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor-4 (HNF-4). J. Biol. Chem. **268:**11125–11133.
26. **Kliewer, S. A., J. M. Lenhard, T. M. Wilson, I. Patel, D. C. Morris, and J. M. Lehmann.** 1995. A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell **83:**813–819.
27. **Ladias, J. A. A., M. Hadzopoulou-Cladaras, D. Kardassis, P. Cardot, J. Cheng, V. Zannis, and C. Cladaras.** 1992. Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3. J. Biol. Chem. **267:**15849–15860.
28. **Lamberts, C., M. Nassal, I. Velhagen, H. Zentgraf, and C. H. Schroder.** 1993. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J. Virol. **67:**3756–3762.
29. **Lazar, M. A.** 1993. Thyroid hormone receptors: multiple forms, multiple possibilities. Endocrine Rev. **14:**184–193.
30. **Lee, H.-J., and C. Chang.** 1995. Identification of human TR2 orphan receptor response element in the transcriptional initiation site of the simian virus 40 major late promoter. J. Biol. Chem. **270:**5434–5440.
31. **Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68:**377–395.
32. **Leng, X., A. J. Cooney, S. Y. Tsai, and M.-J. Tsai.** 1996. Molecular mechanisms of COUP-TF- mediated transcriptional repression: evidence for transrepression and active repression. Mol. Cell. Biol. **16:**2332–2340.
33. **Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans.** 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature **345:**224–229.
34. **Mangelsdorf, D. J., and R. M. Evans.** 1995. The RXR heterodimers and orphan receptors. Cell **83:**841–850.
35. **Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans.** 1995. The nuclear receptor superfamily: the second decade. Cell **83:**835–839.
36. **Okamoto, H., F. Tsuda, Y. Akahane, Y. Sugai, M. Yoshiba, K. Moriyama, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. **68:**8102–8110.
37. **Qiu, Y. H., S. Y. Tsai, and M.-J. Tsai.** 1994. COUP-TF: an orphan member of the steroid/thyroid hormone receptor superfamily. Trends Endocrinol. Metab. **5:**234–239.
38. **Raisher, B. D., T. Gulick, Z. Zhang, A. W. Strauss, D. D. Moore, and D. P. Kelly.** 1992. Identification of a novel retinoid-responsive element in the promoter region of the medium chain acyl-coenzyme A dehydrogenase gene. J. Biol. Chem. **267:**20264–20269.
39. **Raney, A. K., D. R. Milich, A. J. Easton, and A. McLachlan.** 1990. Differentiation-specific transcriptional regulation of the hepatitis B virus large surface antigen gene in human hepatoma cell lines. J. Virol. **64:**2360–2368.
40. **Raney, A. K., J. L. Johnson, C. N. A. Palmer, and A. McLachlan.** 1997. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J. Virol. **71:**1058–1071.
41. **Runkel, L., M. Fischer, and H. Schaller.** 1993. Two-codon insertion mutations of the HBx define two separate regions necessary for its trans-activation function. Virology **197:**529–536.
42. **Sato, S., K. Suzuki, Y. Akahane, K. Akamatsu, K. Akiyama, K. Yunomura, F. Tsuda, T. Tanaka, H. Okamoto, Y. Miyakawa, and M. Mayumi.** 1995. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann. Intern. Med. **122:**241–248.
43. **Schleiss, M. R., C. R. Degnin, and A. P. Geballe.** 1991. Translational control of human cytomegalovirus gp48 expression. J. Virol. **65:**6782–6789.
44. **Sells, M. A., A. Z. Zelent, M. Shvartsman, and G. Acs.** 1988. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. J. Virol. **62:**2836–2844.
45. **Sladek, F. M., W. Zhong, E. Lai, and J. E. Darnell, Jr.** 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. **4:**2353–2365.
46. **Tong, M., M. Thursby, J. Lin, J. Weissman, and C. McPeak.** 1981. Studies on the maternal-infant transmission of the hepatitis B virus and HBV infection within families. Prog. Med. Virol. **27:**137–147.
47. **Tontonoz P., E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman.** 1994. mPPARγ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. **8:**1224–1234.
48. **Tsai, M.-J., and B. W. O'Malley.** 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. **63:**451–486.
49. **Tsai, S. Y., and M.-J. Tsai.** 1997. Chicken ovalbumin upstream promoter transcription factors (COUP-TFs): coming of age. Endocrine Rev. **18:**229–240.
50. **Tur-Kaspa, R., R. D. Burk, Y. Shaul, and D. A. Shafritz.** 1986. Hepatitis B virus DNA contains a glucocorticoid-responsive element. Proc. Natl. Acad. Sci. USA **83:**1627–1631.
51. **Tur-Kaspa, R., Y. Shaul, D. D. Moore, R. D. Burk, S. Okret, L. Poellinger, and D. A. Shafritz.** 1988. The glucocorticoid receptor recognizes a specific nucleotide sequence in hepatitis B virus DNA causing increased activity of the HBV enhancer. Virology **167:**630–633.
52. **Wiley, S. R., R. J. Kraus, and J. E. Mertz.** 1992. Functional binding of the "TATA" box binding component of transcription factor TFIID to the −30 region of TATA-less promoters. Proc. Natl. Acad. Sci. USA **89:**5814–5818.
53. **Wiley, S. R., R. J. Kraus, F. Zuo, E. E. Murray, K. Loritz, and J. E. Mertz.** 1993. SV40 early-to-late switch involves titration of cellular transcriptional repressors. Genes Dev. **7:**2206–2219.
54. **Willy, P. J., K. Umesono, E. S. Ong, R. M. Evans, R. A. Heyman, and D. J. Mangelsdorf.** 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev. **9:**1033–1045.
55. **Yu, X., and J. E. Mertz.** 1996. Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. J. Virol. **70:**8719–8726.
56. **Yu, X., and J. E. Mertz.** Unpublished data.
57. **Zuo, F., and J. E. Mertz.** 1995. Simian virus 40 late gene expression is regulated by members of the steroid/thyroid hormone receptor superfamily. Proc. Natl. Acad. Sci. USA **92:**8586–8590.
58. **Zuo, F., R. J. Kraus, T. Gulick, D. D. Moore, and J. E. Mertz.** 1997. Direct modulation of simian virus 40 late gene expression by thyroid hormone and its receptor. J. Virol. **71:**427–436.