Members of the Nuclear Receptor Superfamily Regulate Transcription from the Hepatitis B Virus Nucleocapsid Promoter†

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The role of members of the nuclear receptor superfamily of transcription factors in regulating hepatitis B virus (HBV) transcription was investigated. Hepatocyte nuclear factor 4 (HNF4), the retinoid X receptor (RXR), and the peroxisome proliferator-activated receptor (PPAR) were examined for their capacity to modulate the level of transcriptional activity from the four HBV promoters by transient-transfection analysis in the dedifferentiated hepatoma cell line, HepG2.1. It was found that the nucleocapsid and large surface antigen promoters were transactivated in the presence of HNF4 whereas the enhancer 1/X gene, nucleocapsid, and large surface antigen promoters were transactivated in the presence of RXR and PPAR. Characterization of the nuclear receptors interacting with the nucleocapsid promoter region demonstrated that HNF4 is the primary transcription factor binding to the regulatory region spanning nucleotides –127 to –102 whereas HNF4, RXR-PPAR heterodimers, COUPTF1, and ARP1 bind the regulatory region spanning nucleotides –34 to –7. Transcriptional transactivation from the nucleocapsid promoter by HNF4 appears to be mediated through the two HNF4 binding sites in the promoter, whereas modulation of the level of transcription from the nucleocapsid promoter by RXR-PPAR appears to be regulated by the regulatory sequence element spanning nucleotides –34 to –7 and the HBV enhancer 1 region. These observations indicate that HBV transcription, and pregenomic RNA synthesis in particular, is regulated by ligand-dependent nuclear receptors. Agonists and antagonists capable of regulating the activity of these nuclear receptors may permit the modulation of HBV transcription and consequently replication during viral infection.

Hepatitis B virus (HBV) is the prototypic member of the hepadnavirus family (16). HBV infection is limited to humans and nonhuman primates and displays a tissue tropism which is restricted primarily to the liver (52). The host range and tissue specificity of HBV infection is probably limited, in part, by the distribution of the putative receptor that the virus uses to enter the hepatocyte. However, it has become apparent from the study of transgenic mice that the liver specificity of HBV transcription is also a major determinant of the viral tissue tropism (1, 14, 19).

The HBV genome is a partially double-stranded 3.2-kb DNA molecule which is converted to covalently closed circular DNA in the nucleus of the infected hepatocyte. The covalently closed circular DNA is the template which is transcribed to generate the 3.5-, 2.4-, 2.1-, and 0.7-kb viral transcripts (3, 4, 28, 66, 73). The nucleocapsid, large surface antigen, major surface antigen, and enhancer 1/X gene promoters direct the expression of these four transcripts, respectively. Each RNA is transcribed from its own promoter and terminates at a common polyadenylation site (16, 52, 72). The structure and function of the four HBV promoters have been quite extensively characterized, and it is apparent that liver-specific expression from the enhancer 1/X gene, nucleocapsid, and large surface antigen promoters results from the combinatorial action of multiple liver-enriched transcription factors, including C/EBP,

HNF1, HNF3, and HNF4, binding to the regulatory sequence elements of these promoters (5, 9, 12, 17, 20, 27, 35, 36, 44, 47, 50, 57, 75, 80). The reason why transcription of the major surface antigen RNA is restricted to hepatocytes *in vivo* is currently unclear, as the identified regulatory sequence elements of the major surface antigen promoter bind ubiquitous transcription factors (51, 61). It is possible that as yet unidentified regulatory elements or the HBV enhancer 1 and 2 regions are responsible for the *in vivo* liver-specific expression of the 2.1-kb RNA (6, 23, 65, 74, 79).

Previous analysis had demonstrated that transcription from the nucleocapsid promoter occurred preferentially in differentiated hepatoma cell lines (22, 54). Detailed analysis of the nucleocapsid promoter identified multiple binding sites for liver-enriched and ubiquitous transcription factors located between –162 and the start site for the precore RNA. The nucleocapsid promoter was shown to include binding sites for transcription factors Sp1 (78), C/EBP (35, 36, 75), HNF3 (27, 34), HNF4 (20), and the TATA-binding protein (8). In addition, a regulatory element located within 30 nucleotides of the transcription start site of the precore RNA appeared to bind an uncharacterized liver-enriched transcription factor (76). In this study, it is shown that multiple members of the nuclear receptor superfamily of transcription factors, including HNF4, retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR), COUPTF1, and ARP1, bind to this regulatory element. Additionally, HNF4 is demonstrated to increase transcription from the nucleocapsid promoter through interactions with either of the HNF4 binding sites located at nucleotide positions –123 to –111 and –28 to –16. In contrast, the RXR-PPAR heterodimer transactivates transcription from the

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FIG. 1. Nucleotide sequence of the HBV nucleocapsid promoter region (subtype ayw) (15). The nucleotide coordinates are derived from the GenBank database, and their position relative to a predominant transcription initiation site (+1; nucleotide coordinate 1785) is shown (22, 23, 60, 69, 71). The underlined sequences represent the Sp1 recognition sequences (78), the HNF3 recognition sequences (27), and the HNF4 recognition sequences (20). The sequences of the HBV double-stranded oligonucleotides CpB, CpC, CpD, CpE, CpFL, and CpHNF4 are indicated. The brackets under the sequence span the regions protected from DNase I digestion by purified recombinant GST-HNF4 and RXR α -MBP/GST-PPAR α . The HBV nucleocapsid promoter HNF4 recognition sequences are shown, and the nucleotides homologous to the nuclear receptor DR1 consensus sequence (41) are indicated (R = A/G; K = G/T; N = A/C/G/T).

nucleocapsid promoter either through the proximal HNF4 regulatory element of the nucleocapsid promoter or through an upstream regulatory element, probably the nuclear receptor binding site in the enhancer 1 region (24, 25, 68). This demonstrates that although HNF4 can bind the promiscuous nuclear receptor regulatory element in the enhancer 1 region (17), it cannot activate transcription from the nucleocapsid promoter from this location in the HBV genome whereas the RXR-PPAR heterodimer appears to have this capability. This implies that different members of the nuclear receptor superfamily binding upstream regulatory elements such as the HBV enhancer 1 region can functionally interact in different ways with the nucleocapsid promoter DNA binding proteins and/or transcription preinitiation complex to modulate RNA synthesis from this promoter.

MATERIALS AND METHODS

Plasmid constructions. The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (58). HBV DNA sequences in these constructions were derived from plasmid pCp10, which contains two copies of the HBV genome (subtype ayw) cloned into the *EcoRI* site of pBR322 (13). The firefly luciferase (LUC) reporter gene in these constructions was derived from plasmid p19DLUC (54). Plasmid CpLUC contains one complete HBV genome located directly 5' to the promoterless LUC reporter gene such that the expression of the LUC gene is governed by the HBV nucleocapsid promoter (54). Similarly, the plasmids SpLUC, XpLUC, and PS1pLUC contain one complete HBV genome located directly 5' to the promoterless LUC reporter gene such that the expression of the LUC gene is governed by the HBV major surface antigen, enhancer 1/X gene, and large surface antigen promoters, respectively. Details of the construction of these plasmids, the nucleocapsid promoter deletion construct Cp Δ 1805-1374LUC and

the cytochrome P-450 4A6 gene promoter construct pLUCA6-880 have been described previously (39, 54, 77).

Plasmids CpM1LUC, CpM2LUC, CpM1M2LUC, Cp Δ 1805-1374M1LUC, Cp Δ 1805-1374M2LUC, and Cp Δ 1805-1374M1M2LUC, containing clustered point mutations, were generated with the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, Calif.) as specified by the manufacturer. The CpM1LUC, CpM2LUC, and CpM1M2LUC plasmids are derivatives of the CpLUC construct, and the Cp Δ 1805-1374M1LUC, Cp Δ 1805-1374M2LUC, and Cp Δ 1805-1374M1M2LUC plasmids are derivatives of the Cp Δ 1805-1374LUC construct. The M1 mutation converted the 13-nucleotide HNF4 binding site 1 sequence located between -123 and -111 (nucleotide coordinates 1662 and 1674) from GGACTCTTGGACT to cGctagctcgtga. The M2 mutation converted the 13-nucleotide HNF4 binding site 2 sequence located between -28 and -16 (nucleotide coordinates 1757 and 1769) from AGGTAAAGGTCT to gaccgctgtagt. The sequences of the mutations introduced into the nucleocapsid promoter constructs were verified by dideoxynucleotide sequencing (59).

Plasmids pHBVTATALUC, CpFL(3)TATALUC, CpFL(4)TATALUC, CpHNF4(3)TATALUC, XpHNF4(2)TATALUC, PS1pHNF1(1)TATALUC, and CpB(3)TATALUC were constructed by inserting synthetic double-stranded oligonucleotides into sites in the polylinker of p19DLUC. pHBVTATALUC was constructed by inserting a double-stranded oligonucleotide containing the large surface antigen promoter TATA-box element, produced by annealing the oligonucleotides CTATATTATATAAGAGAGAAGCT and TCTCTCTTATATAA TATAGGTAC (spanning HBV coordinates 2773 to 2791), into the *SacI* and *KpnI* sites of p19DLUC in the same orientation as the TATA-box element occurs in the HBV genome (49). CpFL(3)TATALUC, CpFL(4)TATALUC, CpHNF4(3)TATALUC, XpHNF4(2)TATALUC, PS1pHNF1(1)TATALUC, and CpB(3)TATALUC were made by inserting one to four copies (as indicated in the construct designation) of the HBV CpFL, CpHNF4, XpHNF4, PS1pHNF1, and CpB (Fig. 1) (49) double-stranded oligonucleotides into the unique *SalI* site or *HindIII* site of pHBVTATALUC. The oligonucleotide pairs used to generate the CpFL, CpHNF4, XpHNF4, PS1pHNF1, and CpB double-stranded oligonucleotides were TCGAGATTAGGTAAAGGTCTTTGTAC TAG and TCGACTAGTACAAAGACCTTTAACCTAATC (oligonucleotide CpFL, HBV coordinates 1751 to 1778), TCGAAAGAGGACTCTTGGACTCT

CAGCAAT and TCGAATTGCTGAGAGTCCAAGAGTCTCTT (oligonucleotide CpHNF4, HBV coordinates 1658 to 1683), TCGATACCTGAACCTT TACCCCGTTGCCCG and TCGACGGGCAACGGGGTAAAGGTTTCAG GTA (oligonucleotide XpHNF4, HBV coordinates 1133 to 1159), AGCTAGT TAATCATTACTTC and AGCTGAAGTAATGATTAAC (oligonucleotide PS1pHNF1, HBV coordinates 2719 to 2734), and TCGACTGGGAGGAGTT and TCGAAACTCTCCCAAG (oligonucleotide CpB, HBV coordinates 1730 to 1743). The CpFL, CpHNF4, XpHNF4, PS1pHNF1, and CpB double-stranded oligonucleotides span the nucleocapsid promoter HNF4 binding sites 2 and 1 (Fig. 1) (20), the enhancer 1/X gene promoter HNF4 binding site (17), the large surface antigen HNF1 binding site (50), and the nucleocapsid promoter Sp1 site 2 (78), respectively. The sequence of each construct was verified by dideoxynucleotide sequencing (59).

The pCMVHNF4, pRS-hRXR α , and pCMVPPAR α -G vectors express HNF4, RXR α , and PPAR α -G polypeptides from the rat HNF4, human RXR α , and mouse PPAR α -G cDNAs, respectively, by using the cytomegalovirus immediate-early promoter (pCMV) or the Rous sarcoma virus long terminal repeat (pRS) (7, 38, 39). The PPAR α -G polypeptide contains a mutation in the PPAR α cDNA changing Glu²⁸² to Gly, which may decrease the affinity of the receptor for the endogenous ligand. Consequently, this mutation increases the peroxisome proliferator-dependent (i.e., clofibrate acid-dependent) activation of transcription from a peroxisome proliferator response element (PPRE)-containing promoter (39) and was used in this study to demonstrate the peroxisome proliferator-dependent transcriptional transactivation of the nucleocapsid promoter. The pGEXHNF4, pGEXRXR α , and pGEXPPAR α vectors express glutathione S-transferase (GST) fusion proteins in *Escherichia coli* DH5 α which include the complete HNF4, RXR α , and PPAR α polypeptide sequences (63). Expression and subsequent affinity purification of the GST fusion proteins with glutathione-agarose was performed as described previously (63). Expression and subsequent affinity purification of the RXR α maltose-binding protein was performed as previously described (46).

Cells and transfections. The human hepatoma cell lines Huh7 (40) and HepG2.1 (54) were grown in RPMI 1640 medium plus 10% fetal bovine serum at 37°C in 5% CO₂-air. The human cervical carcinoma cell line HeLa S3 (48) was grown in Dulbecco's modified Eagle's medium containing 4.5 mg of glucose per ml and 10% calf serum at 37°C in 5% CO₂-air. Transfections were performed as previously described (18, 64), except that six-well plates, containing approximately 3×10^5 cells, were used. The transfected DNA mixture comprised 5 μ g of a LUC plasmid and 0.25 μ g of pCMV β , which served as an internal control for transfection efficiency. pCMV β directs the expression of the *E. coli* β -galactosidase gene by using the cytomegalovirus immediate-early promoter (Clontech Laboratories, Palo Alto, Calif.). When appropriate, the DNA mixture also included 0.5 μ g of the HNF4, RXR α , and PPAR α -G expression vectors pCMVHNF4, pRS-hRXR α , and pCMVPPAR α -G, respectively, or the control expression vectors pCMV and pRS. The DNA was removed 16 to 24 h after transfection and washed with 2 ml of medium. Fresh RPMI 1640 medium containing all-trans retinoic acid and clofibrate acid at final concentrations of 1 μ M and 1 mM, respectively, was added to the cells as required and incubated for 24 h before being replaced with the same medium for an additional 24 h. Cell extracts were prepared 40 to 48 h after transfection when the pCMVHNF4 expression vector was being analyzed and 64 to 72 h after transfection when the pRS-hRXR α and pCMVPPAR α -G expression vectors were being analyzed. Cells were lysed in 150 μ l of lysis buffer (0.1 M potassium phosphate [pH 7.8], 0.2% [vol/vol] Triton X-100), and the cell debris was pelleted by centrifugation for 2 min at 13,000 rpm in a Biofuge 13 microcentrifuge. The supernatant was assayed for LUC activity essentially as previously described (10) and for β -galactosidase activity with a Galacto-Light kit (Tropix, Inc.) as instructed by the manufacturer. The level of β -galactosidase activity observed was not specifically affected by any of the exogenously expressed transcription factors. The LUC activities were normalized to the level of β -galactosidase activity in each transfection experiment.

Whole-cell extracts, gel retardation analysis, and DNase I footprinting. Nuclear extracts were prepared from Huh7, HepG2.1, and HeLa S3 cells and mouse liver, as described previously (11, 55, 62). Gel retardation analysis was performed essentially as described previously (50). ³²P-labeled double-stranded oligonucleotide (1 ng) (see above) was incubated with 9 μ g of nuclear extract prior to polyacrylamide gel electrophoresis (4% polyacrylamide) and autoradiography. When gel retardation competition analysis was performed, the cell extract was preincubated with 750 ng of cold double-stranded competitor oligonucleotide for 15 min prior to the addition of the ³²P-labeled double-stranded oligonucleotide. Gel retardation analysis with antibodies was performed essentially as described previously (32). The cell extract was incubated with 1 μ l of antiserum specific for the COUPTF1, ARP1, HNF4, RXR, and PPAR polypeptides (29, 45, 62, 70) for 1 h prior to the addition of the ³²P-labeled double-stranded oligonucleotide. Additional double-stranded oligonucleotides used in gel shift analysis included the PPRE from the rabbit cytochrome P-450 4A6 promoter, CGCAAACTG AACTAGGGCAAAGTTGAGGGCAG (oligonucleotide 4A6-Z, -677 to -644 relative to the transcription initiation site) (39, 46); a nuclear receptor perfect direct repeat sequence with a spacing between repeats of one nucleotide, ACA AACTAGGTCAAAGGTCAGGG (oligonucleotide DRIP) (45); and the PPRE from the rat mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) synthase promoter, CAGAAAAGTGGGCCAAAGGTCTCAG (oligo-

TABLE 1. Relative activity of HBV promoters in HepG2.1 cells with and without exogenously expressed HNF4 polypeptide^a

Promoter ^b	Relative activity		Fold induction
	-HNF4	+HNF4	
SpLUC	1.00 (0.05)	1.88 (0.55)	1.9
XpLUC	2.40 (0.54)	2.62 (0.71)	1.1
CpLUC	0.39 (0.22)	1.27 (0.75)	3.3
PS1pLUC	0.011 (0.009)	0.10 (0.04)	9.1

^a The activities of the HBV promoters are reported relative to the activity of the major surface antigen promoter (Sp) in the absence of exogenously expressed HNF4. The standard deviation of the mean is shown in parentheses. The expression vectors used were pCMV (-HNF4) and pCMVHNF4 (+HNF4).

^b Xp, X gene promoter; Cp, nucleocapsid promoter; PS1p, large surface antigen promoter.

nucleotide HMG, -107 to -82 relative to the transcription initiation site) (45). The control double-stranded oligonucleotides derived from the nucleocapsid promoter, oligonucleotide CpC (Sp1 site 3), oligonucleotide CpD, and oligonucleotide CpE (HNF3 site 2) (Fig. 1) and the control double-stranded oligonucleotides derived from the major surface antigen promoter, oligonucleotide C, oligonucleotide D (Sp1 site 2), oligonucleotide E, and oligonucleotide G, have been described previously (27, 51).

The DNase I footprinting assay was performed as described previously (2, 51, 55). The reaction mixtures contained 1 to 5 ng of end-labeled DNA fragment in 100 μ l containing 10 mM Tris hydrochloride (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% (vol/vol) glycerol, and purified recombinant GST-HNF4 or RXR α /MBP plus GST-PPAR α polypeptide. Binding was carried out for 15 min at 0°C and then for 2 min at room temperature, after which 100 μ l of 5 mM CaCl₂-10 mM MgCl₂ containing DNase I was added at room temperature. The reaction mixture was incubated for 2 min, after which the reaction was stopped by the addition of 200 μ l of 1% (wt/vol) sodium dodecyl sulfate-20 mM EDTA-200 mM NaCl containing 125 μ g of tRNA per ml. The mixture was precipitated with ethanol and analyzed by 6% urea-acrylamide sequencing gel electrophoresis and autoradiography.

RESULTS

Previous studies had identified a promoter-proximal sequence of the nucleocapsid minimal promoter located within the -28 to -7 region as an important regulatory element for transcription from this promoter in the differentiated hepatoma cell line, Huh7 (76). This promoter sequence contains an element which displays strong homology to two copies of the direct-repeat (DR) sequence, (A/G)G(G/T)TCA, separated by a single nucleotide (Fig. 1) (41). This type of sequence is typical of the nuclear receptor response element, DR1, which has been shown to interact with homodimeric nuclear receptors including HNF4, COUPTF1, ARP1, and RXR and with RXR heterodimers including RXR-PPAR (30, 37, 41).

Influence of nuclear receptors on transcription from the four HBV promoters. The influence of the liver-enriched transcription factor, HNF4, on the transcriptional activities of the four HBV promoters was measured in the dedifferentiated hepatoma cell line HepG2.1 by transient-transfection analysis. Exogenous expression of the HNF4 polypeptide increased the level of transcription from the large surface antigen, the nucleocapsid, and the major surface antigen promoters approximately nine-, three-, and twofold, respectively, whereas HNF4 does not appear to affect the level of transcription from the enhancer 1/X gene promoter (Table 1). For comparison, the effect of exogenous expression of the RXR α -PPAR α heterodimeric protein in the presence or absence of retinoic acid and clofibrate acid was examined (Table 2). Retinoic acid is converted intracellularly to 9-cis-retinoic acid, which is the RXR ligand (21, 33). Clofibrate acid may act directly as a ligand for PPAR α or may stimulate the transcriptional activity of PPAR α by increasing the intracellular concentration of an

TABLE 2. Relative activity of HBV promoters in HepG2.1 cells with and without exogenously expressed RXR α and PPAR α polypeptides in the presence or absence of retinoic acid and clofibrilic acid^a

Promoter ^b	Relative activity				Fold induction ^c
	-R-L	-R+L	+R-L	+R+L	
SpLUC	1.00 (0.00)	1.09 (0.21)	1.36 (0.40)	1.40 (0.53)	1.4
XpLUC	4.42 (1.02)	3.24 (0.45)	2.99 (0.59)	9.39 (0.91)	2.1
CpLUC	1.31 (0.17)	1.92 (0.20)	2.19 (0.35)	4.90 (0.42)	3.7
PS1pLUC	0.025 (0.005)	0.044 (0.010)	0.043 (0.011)	0.095 (0.020)	3.8

^a The activities of the HBV promoters are reported relative to the activity of the major surface antigen promoter (Sp) in the absence of exogenously expressed RXR α and PPAR α . The standard deviation of the mean is shown in parentheses. The nuclear receptor expression vectors used were pRS plus pCMV (-R) and pRS-hRXR α plus pCMVPPAR α -G (+R). HepG2.1 cells were incubated in the absence (-L) or presence (+L) of the 1 μ M retinoic acid plus 1 mM clofibrilic acid.

^b Xp, X gene promoter; Cp, nucleocapsid promoter; PS1p, large surface antigen promoter.

^c Fold induction represents the ratio of the relative activity in the presence of receptors and ligands (+R+L) to the relative activity in the absence of receptors and ligands (-R-L).

unidentified endogenous ligand for this nuclear receptor (26). RXR α -PPAR α increases the level of transcription from the large surface antigen, the nucleocapsid, and the enhancer 1/X gene promoters approximately four-, four-, and twofold, respectively, whereas it does not appear to affect greatly the level of transcription from the major surface antigen promoter (Table 2). In each case, addition of retinoic acid plus clofibrilic acid or nuclear receptors alone had a very limited effect on transcription from the HBV promoters. It appears that both the receptors and the ligands are required for the maximal increase in transcription from the HBV promoters.

Identification of the nuclear receptor binding sites in the nucleocapsid promoter. A previous study had located an HNF4 binding site in the nucleocapsid promoter sequence between -135 and -110 by methylation interference analysis (20). This region also contains a DR1 sequence element which probably represents the HNF4 recognition sequence (Fig. 1). To confirm this observation and to examine the nucleocapsid promoter for additional HNF4 recognition elements, DNase I footprinting analysis with a purified recombinant GST-HNF4 polypeptide was performed (Fig. 2). Two HNF4 footprints spanning the promoter regions from -132 to -102 (HNF4 site 1) and from -34 to -5 (HNF4 site 2) were observed. A 13-nucleotide region homologous to the nuclear receptor DR1 recognition sequence was located within both footprinted regions (Fig. 1).

As transcription from the nucleocapsid promoter was increased by the expression of the RXR α and PPAR α polypeptides (Table 2), DNase I footprinting analysis was performed with purified recombinant RXR α -MBP/GST-PPAR α heterodimers to examine the location of the PPRE in the nucleocapsid promoter. In contrast to the result obtained with HNF4, a single RXR α -PPAR α footprint was observed spanning the promoter region from -32 to -6 (Fig. 3). These observations suggest that the HNF4 site 1 selectively binds the HNF4 polypeptide whereas the HNF4 site 2 region interacts with both HNF4 and the heterodimeric nuclear receptor, RXR α -PPAR α .

To characterize further the binding of the purified recombinant RXR α and PPAR α polypeptides to HNF4 site 2 in the nucleocapsid promoter, gel shift analysis was performed with the double-stranded oligonucleotide, CpFL, spanning HNF4 site 2 (Fig. 1). The binding of the RXR α and PPAR α polypeptides to a perfect direct repeat (DR1P) sequence, AGGTCA AAGGTCA (45), and a PPRE (4A6-Z) from the cytochrome P-450 4A6 gene (46), was compared to the binding of these nuclear receptors to HNF4 site 2 (CpFL) sequence (Fig. 4). The RXR α polypeptide bound to the DR1P double-stranded oligonucleotide, presumably as a homodimer, whereas the

PPAR α polypeptide did not form a complex with the DR1P sequence (Fig. 4A) (45). The gel shift complex formed between the DR1P sequence and the RXR α and PPAR α polypeptides migrated more slowly than the complex formed with the RXR α alone, indicating that this complex represents the

FIG. 2. DNase I footprinting analysis of the long (minus) strand of the HBV nucleocapsid promoter (lanes 1 and 2). A 1,119-nucleotide HBV DNA fragment from -1099 to +20 was 5'-end labeled at +20 and incubated in the absence (lane 1) or presence (lane 2) of purified recombinant GST-HNF4. DNase I footprinting analysis of the short (plus) strand of the HBV nucleocapsid promoter (lanes 3 and 4). A 1,119-nucleotide HBV DNA fragment from -1099 to +20 was 3'-end labeled at +20 and incubated in the absence (lane 3) or presence (lane 4) of purified recombinant GST-HNF4. The upper and lower HNF4 footprints are designated HNF4 sites 1 and 2, respectively (Fig. 1). An unrelated DNA sequence (GATC) was used as a size standard to locate the HNF4 binding sites.

FIG. 3. DNase I footprinting analysis of the short (plus) strand of the HBV nucleocapsid promoter. A 1,119-nucleotide HBV DNA fragment from -1099 to $+20$ was 3'-end labeled at $+20$ and incubated in the absence (lane 1) or presence (lane 2) of purified recombinant RXR α -MBP/GST-PPAR α . The RXR α -PPAR α footprint region is indicated in Fig. 1. An unrelated DNA sequence (GATC) was used as a size standard to locate the HNF4 binding sites.

RXR α -PPAR α heterodimer bound to the DR1P sequence. With the 4A6-Z PPPE, no specific complexes were formed when the RXR α and PPAR α polypeptides were examined individually (Fig. 4B) (45). In the presence of both polypeptides, the RXR α -PPAR α heterodimer bound to the 4A6-Z sequence as expected (45, 46). In the case of the HNF4 site 2 CpFL double-stranded oligonucleotide, no complexes were observed with the RXR α polypeptide alone (Fig. 4C). The PPAR α polypeptide appears to form a specific complex with the CpFL double-stranded oligonucleotide. This was unexpected, as the PPAR α polypeptide normally binds PPPE as a heterodimer with RXR (45). The significance of this complex is uncertain. However, in the presence of both the RXR α and PPAR α polypeptides, the RXR α -PPAR α heterodimer complex appears as the predominant complex with CpFL, confirming that this heterodimeric nuclear receptor binds to this nucleocapsid regulatory element. Consistent with the DNase I footprinting analysis, the RXR α -PPAR α heterodimer failed to demonstrate the formation of a complex with the CpHNF4 double-stranded oligonucleotide, CpHNF4, spanning HNF4 site 1 (53). This observation further supports the contention that HNF4 site 1 is less promiscuous than HNF4 site 2 in its binding of nuclear receptors.

Characterization of the DNA binding proteins in nuclear extracts which interact with the HBV nucleocapsid promoter HNF4 binding sites. DNase I footprinting analysis has demonstrated that purified recombinant HNF4 binds to the HNF4 sites 1 and 2 in the nucleocapsid promoter whereas the RXR α -PPAR α heterodimer binds to HNF4 site 2 but not to HNF4 site 1. However, the analysis with the recombinant polypeptides does not indicate the nature of the factor(s) in human hepatoma cell lines which interacts with these regulatory elements. To examine this issue, double-stranded oligonucleotides spanning HNF4 sites 1 and 2 of the nucleocapsid promoter (CpHNF4 and CpFL, respectively [Fig. 1]) and the previously described nuclear receptor binding site in the enhancer 1/X gene promoter (XpHNF4) (17, 24, 25) were characterized for their ability to bind proteins present in Huh7 nuclear extracts by gel supershift analysis (Fig. 5). Each of the three HBV sites bound proteins present in the Huh7 nuclear extracts, demonstrating that these regulatory elements are recognized by transcription factors present in this cell line. In addition, an antibody to HNF4 generated a supershifted complex with the nuclear proteins recognizing each of these regulatory elements (Fig. 5, lanes 2, 5, and 8). This observation demonstrated that HNF4 present in Huh7 nuclear extracts interacts with each of these three HBV regulatory sequence elements. In the case of the CpHNF4 double-stranded oligonucleotide, almost all of the complex formed with the Huh7 nuclear extract was supershifted by the HNF4 antibody, indicating this is the primary transcription factor interacting with this regulatory element. In contrast, only a portion of the DNA-protein complexes formed between the XpHNF4 and CpFL double-stranded oligonucleotides was supershifted by the HNF4 antibody, indicating that additional members of the nuclear receptor superfamily of transcription factors bind to these recognition elements. In the case of the XpHNF4 double-stranded oligonucleotide, this observation is consistent with previous observations that this regulatory element also binds RXR α , PPAR, and COUPTF1 (17, 24, 25).

In an attempt to characterize further the members of the nuclear receptor superfamily of transcription factors which can interact with the CpFL regulatory element of the nucleocapsid promoter, additional antibodies were examined for their ability to supershift the complexes formed between the CpFL double-stranded oligonucleotide and the factors present in nuclear extracts prepared from Huh7 cells, HepG2.1 cells, and mouse hepatocytes (Fig. 6). In the case of Huh7 and HepG2.1 nuclear extracts, CpFL appears to form two closely migrating complexes comprising a major faster-migrating complex and a minor slower-migrating complex. The antibody to COUPTF1 appears to supershift the majority of the slower-migrating complex, whereas the antibody to ARP1 appears to supershift the majority of the faster-migrating complex (Fig. 6A and B, lanes 2 and 3). Therefore, it appears that the majority of the complexes formed between CpFL and the Huh7 and HepG2.1 nuclear extracts are comprised of COUPTF1 and ARP1. In the case of the Huh7 nuclear extract, the antibody to HNF4 also generates a supershifted complex, demonstrating that HNF4 is present in the Huh7 nuclear extract but appears to be absent from the HepG2.1 nuclear extract (Fig. 5 and 6A and B, lane 4). The antibodies to RXR and PPAR failed to generate observable supershifted complexes, indicating that these nuclear receptors were not major components of the complexes formed between the CpFL double-stranded oligonucleotide and the Huh7 and HepG2.1 nuclear extracts (Fig. 6A and B, lanes 5 and 6). In an attempt to determine if additional nuclear receptors interact with the CpFL double-stranded oligonucleotide, the complexes formed with mouse liver nuclear extracts

FIG. 4. Gel retardation and complex inhibition analysis of the nuclear receptor perfect direct repeat 1 sequence (DR1P) (A), the PPRE Z sequence element from the cytochrome P-450 4A6 promoter (4A6-Z) (B), and the HBV nucleocapsid promoter CpFL regulatory sequence element (C) with purified recombinant GST-RXR α and GST-PPAR α . The 32 P-labeled, double-stranded oligonucleotides DR1P (A), 4A6-Z (B), and CpFL (C) and purified recombinant GST-RXR α (RXR; lanes 1 and 2), GST-PPAR α (PPAR; lanes 3 and 4), and GST-RXR α plus GST-PPAR α (RXR+PPAR; lanes 5 and 6) were used for this analysis. The unlabeled, double-stranded oligonucleotide CpFL (Fig. 1) was used as competitor DNA to demonstrate the specificity of the observed complex (lanes 2, 4, and 6).

were analyzed (Fig. 6C). Major supershifted complexes were observed with antibodies to ARP1, HNF4, and RXR (the mRXR monoclonal antibody produced a more pronounced supershifted complex than the anti-peptide RXR antiserum),

FIG. 5. Gel retardation and complex supershift analysis with an HNF4-specific antiserum. The 32 P-labeled, double-stranded oligonucleotides, CpFL (lanes 1 to 3), XpHNF4 (lanes 4 to 6), and CpHNF4 (lanes 7 to 9) and Huh7 nuclear extracts were used for this analysis. Anti-HNF4 antibodies (HNF4; lanes 2, 5, and 8) and control anti-HNF3 α antibodies (HNF3; lanes 3, 6, and 9) were included as indicated.

whereas minor supershifted complexes were seen with antibodies to COUPTF1 and PPAR, indicating that each of these nuclear receptors was present in the mouse liver nuclear extract and bound to the CpFL regulatory element. Based on this analysis, it appears that there may be additional nuclear receptors present in the mouse liver which bind to the CpFL element, as the total amount of supershifted complexes appears to represent only a proportion of the total complex formed between the mouse liver nuclear extract and the CpFL double-stranded oligonucleotide.

To examine the specificity of the complexes formed between the CpFL double-stranded oligonucleotide and the nuclear extracts, several unlabeled, double-stranded oligonucleotides were used as competitors in the gel retardation analysis (Fig. 7). Unlabeled, double-stranded oligonucleotides CpC (Sp1 site 3), CpD, and CpE (HNF3 site 2) from the nucleocapsid promoter and C, D (Sp1 site 2), E, and G from the major surface antigen promoter failed to compete efficiently for the factors binding the CpFL double-stranded oligonucleotide. The unlabeled nucleocapsid promoter CpFL double-stranded oligonucleotide and the PPRE from the rat mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase promoter efficiently competed with the 32 P-labeled CpFL double-stranded oligonucleotide for binding the nuclear receptors in the nuclear extract from Huh7 cells, HepG2.1 cells, HeLa S3, and mouse liver cells. These results are consistent with the CpFL regulatory element specifically binding multiple members of the nuclear receptor superfamily of transcription factors known to interact with PPREs, including the ubiquitous factors, COUPTF1 and ARP1, and the liver-enriched factors, HNF4, RXR α , and PPAR α .

HNF4 transactivates expression from the HBV nucleocapsid promoter through both HNF4 binding sites 1 and 2. Exogenously expressed HNF4 transactivates transcription from the nucleocapsid promoter approximately threefold in the con-

FIG. 6. Gel retardation and complex supershift analysis of the nuclear receptors interacting with the CpFL regulatory element of the nucleocapsid promoter. The 32 P-labeled, double-stranded oligonucleotide CpFL and Huh7 nuclear extract (H7) (A), HepG2.1 nuclear extract (2.1) (B), and mouse liver nuclear extract (ML) (C) were used for this analysis. Anti-COUPTF1 antibodies (COUPTF1; lanes 2), anti-ARP1 antibodies (ARP1; lanes 3), anti-HNF4 antibodies (HNF4; panels A and B, lanes 4; panel C, lane 7), anti-RXR α antibodies (RXR; panels A and B, lanes 5; panel C, lane 8), anti-RXR monoclonal antibody (mRXR; panel C, lane 9), anti-PPAR antibodies (PPAR; panels A and B, lanes 6; panel C, lane 4), and preimmune (PI) serum were included as indicated.

struct CpLUC which contains the complete HBV genome located upstream of the luciferase reporter gene (Table 1; Fig. 8). To define the role of the HNF4 binding sites in the activation of transcription from the nucleocapsid promoter, clustered point mutations were introduced into HNF4 sites 1 (mutation M1) and 2 (mutation M2) and examined for their effect on HNF4-mediated transcriptional transactivation from the nucleocapsid promoter in HepG2.1 transient-transfection analysis (Fig. 8). Mutation of the individual HNF4 binding sites alone did not eliminate the HNF4-mediated increase in transcription from the nucleocapsid promoter. Mutation of HNF4 site 1 reduced the induction of transcription to 2.4-fold whereas mutation of HNF4 site 2 increased the induction of transcription to 5-fold from the nucleocapsid promoter in response to exogenously expressed HNF4 (Fig. 8). This result suggests the possibility that the transcription factors interacting with HNF4 site 2 actually limit the capacity of HNF4 interacting with HNF4 site 1 to activate transcription from the nucleocapsid promoter. The simultaneous mutation of both HNF4 sites 1 and 2 eliminates HNF4-mediated transcriptional transactivation from the nucleocapsid promoter. This indicates that both these sites are involved in increasing the level of transcription from the nucleocapsid promoter by HNF4 in the context of the complete HBV genome.

Mutational analysis of the nucleocapsid promoter indicated that HNF4 sites 1 and 2 were the functional regulatory elements responsible for mediating HNF4 transcriptional transactivation of the nucleocapsid promoter. To verify further the functional properties of the nucleocapsid promoter HNF4 binding sites, they were examined for their capacity to mediate transcriptional transactivation by HNF4 in the context of a TATA-box element. HNF4 site 1 and 2 double-stranded oli-

gonucleotide synthetic promoter elements were cloned upstream of a TATA-box element and the LUC open reading frame (ORF) and were tested for their transcriptional activities in HepG2.1 cells in the presence or absence of the HNF4 expression vector (Fig. 9). In the absence of the HNF4 expression vector, the constructs containing the nucleocapsid promoter HNF4 sites 1 [construct CpHNF4(3)LUC] and 2 [constructs CpFL(3)LUC and CpFL(4)LUC] had very little transcriptional activity. When the HNF4 polypeptide was exogenously expressed, the synthetic constructs containing HNF4 sites 1 and 2 were transcriptionally activated to a level similar to that observed for the HNF4 binding site in the HBV enhancer 1/X gene promoter [construct XpHNF4(2)LUC] (17). In contrast, transcription from the large surface antigen promoter HNF1 binding site [PS1pHNF1(1)LUC] and nucleocapsid promoter Sp1 site 2 [construct CpB(3)LUC] were not activated by the exogenous expression of the HNF4 transcription factor. Therefore, the oligonucleotides containing the nucleocapsid promoter HNF4 binding sites appear to be functional sites which can mediate specific transcriptional transactivation by HNF4 both in the context of a TATA-box sequence and in the context of the nucleocapsid promoter.

RXR α -PPAR α transactivates expression from the HBV nucleocapsid promoter through an upstream regulatory region and HNF4 binding site 2. Exogenously expressed RXR α -PPAR α transactivates transcription from the nucleocapsid promoter in a ligand-dependent manner (Table 2). Maximal transcriptional transactivation from the nucleocapsid promoter is dependent on the presence of both the RXR α and PPAR α nuclear receptors and their respective ligands (Fig. 10) in a manner which is very similar to the previously characterized PPRE-containing cytochrome P-450 4A6 promoter (39, 46). In

FIG. 7. Gel retardation and complex inhibition analysis of the HBV nucleocapsid promoter CpFL regulatory sequence element. The 32 P-labeled, double-stranded oligonucleotide CpFL (Fig. 1) and Huh7 nuclear extract (A), HepG2.1 nuclear extract (B), HeLa S3 nuclear extract (C), and mouse liver nuclear extract (D) were used for this analysis. Unlabeled, double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. CpC (lanes 2), nucleocapsid promoter Sp1 binding site 3 (78); CpD (lanes 3), nucleocapsid promoter control oligonucleotide (78); CpE (lanes 4), nucleocapsid promoter HNF3 binding site 2 (27); CpFL (lanes 5), nucleocapsid promoter nuclear receptor binding site; HMG (lanes 6), 3-hydroxy-3-methylglutaryl-CoA synthase promoter PPRE; C (lanes 7), major surface antigen promoter regulatory element (56); D (lanes 8), major surface antigen promoter Sp1 site 2 binding site (51); E (lanes 9), major surface antigen promoter regulatory element (56); G (lanes 10), major surface antigen promoter regulatory element (56).

the case of the nucleocapsid promoter, it is apparent that addition of retinoic acid and clofibric acid does not increase the level of transcription from the nucleocapsid promoter in HepG2.1 cells when assayed by transient-transfection analysis in the absence of exogenously expressed nuclear receptors. Similarly, exogenous expression of the RXR α and PPAR α nuclear receptors without the addition of retinoic acid or clofibric acid did not affect the level of transcription from the nucleocapsid promoter. Maximal induction of the nucleocapsid promoter was observed when both receptors and retinoic acid plus clofibric acid were introduced into the HepG2.1 cells. However, an intermediate level of induction of the nucleocapsid promoter was observed when RXR α was introduced into HepG2.1 cells in the presence of retinoic acid. As RXR α homodimers appear not to bind to either the enhancer 1/X gene promoter nuclear receptor binding site (24) or the nucleocapsid promoter HNF4 site 2 (Fig. 4), this effect is probably due to RXR α forming a heterodimer with an endogenous intracellular nuclear receptor present in HepG2.1 cells which is capable of binding to a DR1 nuclear receptor response element.

To define the role of the HNF4 binding sites in the activation of transcription from the nucleocapsid promoter, clustered point mutations in HNF4 sites 1 (mutation M1) and 2 (mutation M2) were examined for their effect on RXR α -

PPAR α -mediated transcriptional transactivation from the nucleocapsid promoter by HepG2.1 transient-transfection analysis in the context of the complete HBV genome (Fig. 11). Mutation of the individual HNF4 binding sites 1 and 2 alone or in combination did not eliminate transcriptional induction from the nucleocapsid promoter by the nuclear receptors in the presence of their ligands. This is in contrast to the results of the HNF4-mediated transcriptional transactivation and suggests that the PPRE in the enhancer 1/X gene promoter region may be responsible for mediating the increase in transcription from the nucleocapsid promoter in the absence of the functional HNF4 sites 1 and 2 (construct CpM1M2LUC). In addition, it was observed that mutation of HNF4 site 1 resulted in enhanced transcriptional transactivation from the nucleocapsid promoter by the RXR α -PPAR α nuclear receptors. This suggests that factors binding to the HNF4 site 1 might prevent maximal increases in transcription from the nucleocapsid promoter mediated by RXR α -PPAR α heterodimers.

To examine further the role of the nucleocapsid promoter HNF4 binding sites 1 and 2 in mediating the RXR α -PPAR α transcriptional transactivation from the nucleocapsid promoter, the M1 and M2 mutations were examined in the absence of the enhancer 1/X gene promoter region (constructs Cp Δ 1805-1374LUC, Cp Δ 1805-1374M1LUC, Cp Δ 1805-1374M2LUC and Cp Δ 1805-1374M1M2LUC) (Fig. 11). In this analysis, it

FIG. 8. Clustered point mutational analysis of the nucleocapsid promoter. Relative activities of the clustered point mutation constructs in HepG2.1 cells in the absence (-HNF4) or presence (+HNF4) of exogenously expressed HNF4 polypeptide, using the expression vectors pCMV and pCMVHNF4, respectively, are indicated. The activities were reported relative to the activity of the full-length promoter construct, CpLUC, in the absence of exogenously expressed HNF4 polypeptide. The standard deviation of the mean is shown in parentheses. The internal control used to correct for transfection efficiencies was pCMV β . The diagrammatic representation of the construct CpLUC is shown. Arrows indicate the positions and directions of transcription from the HBV large surface antigen (PSp), major surface antigen (Sp), enhancer 1/X gene (Xp), core (Cp), or nucleocapsid promoters, respectively. Boxes indicate the positions of the HBV enhancer 1 sequence (Eh), HBV polyadenylation sequence (pA), X gene ORF (X), presurface antigen ORF (PS), surface antigen ORF (S), precore ORF (PC), core ORF (C), polymerase ORF (P), and LUC ORF (LUC). The horizontal lines indicate the HBV sequences present in the CpLUC plasmids. Plasmid CpLUC contains the HBV sequences from nucleotide coordinates 1805 to 3182/1 to 1804 (nucleotide sequences are designated by using coordinates derived from the GenBank genetic sequence data bank). The locations of the M1 and M2 mutations are indicated by solid boxes.

was apparent that the M2 mutation, which alters the HNF4 site 2, prevents the RXR α -PPAR α -mediated transcriptional transactivation of the nucleocapsid promoter. This is consistent with the observation that the RXR α -PPAR α heterodimer binds to HNF4 site 2 but does not interact with HNF4 site 1 (Fig. 1 and 3). Consequently, it appears that the enhancer 1/X gene pro-

motor region containing a DR1 nuclear receptor binding site and nucleocapsid HNF4 site 2 increase the level of transcription from the nucleocapsid promoter in the presence of the RXR α and PPAR α nuclear receptors and their ligands.

To confirm that nucleocapsid HNF4 site 2 and the enhancer 1/X gene promoter nuclear receptor DR1 binding site can

FIG. 9. Functional analysis of the HBV nucleocapsid promoter HNF4 binding sites. The constructs examined contain the CpFL (nucleocapsid promoter HNF4 site 2), CpHNF4 (nucleocapsid promoter HNF4 site 1), XpHNF4 (enhancer 1/X gene promoter HNF4 site), PS1pHNF1 (large surface antigen promoter HNF1 site), and CpB (nucleocapsid promoter Sp1 site 2) double-stranded oligonucleotides cloned into the minimal promoter construct, pHBVTATALUC (see the insert). The number of copies of the oligonucleotide in the construct is shown in parentheses in the construct name. The relative activities of the constructs in the absence (-HNF4) (□) or presence (■) (+HNF4) of exogenously expressed HNF4, using the expression vector, pCMV and pCMVHNF4, respectively, were examined in HepG2.1 cells. The transcriptional activities are reported relative to the CpFL(3)LUC plasmid in the presence of the HNF4 expression vector, which is designated as having a relative activity of 1.0. The standard deviation of the mean is indicated by an error bar. The internal control used to correct for transfection efficiencies was pCMV β .

FIG. 10. Ligand-dependent transcriptional transactivation of the nucleocapsid promoter by RXR α and PPAR α . The relative activities of cytochrome P-450 4A6 promoter construct (pLUCA6-880) and the nucleocapsid promoter construct (CpLUC) were examined in the presence of retinoic acid (+RA), clofibrate acid (+CA), RXR α (+RXR), and PPAR α (+PPAR) as indicated. The transcriptional activities are reported relative to the pLUCA6-880 plasmid (relative activity = 1.0) in the absence of ligands or nuclear receptors (no additions). The internal control used to correct for transfection efficiencies was pCMV β .

mediate RXR α -PPAR α -dependent transcriptional transactivation, these regulatory elements were examined in the context of a TATA-box-containing minimal promoter (Fig. 12). In this analysis, it is apparent that nucleocapsid promoter HNF4 site 2 [constructs CpFL(3)LUC and CpFL(4)LUC] and the enhancer 1/X gene promoter HNF4 site [construct XpHNF4(2)LUC] can mediate ligand-dependent transcriptional transactivation by the RXR α -PPAR α nuclear receptors from a minimal promoter. In contrast, nucleocapsid promoter HNF4 site 1 [construct CpHNF4(3)LUC] failed to mediate ligand-dependent transcriptional transactivation by the RXR α -PPAR α nuclear receptors from a minimal promoter. This result is consistent with the observation that this regulatory element binds HNF4 but does not bind the RXR α -PPAR α heterodimer. The control HNF1 binding site from the large surface antigen promoter and the Sp1 site 2 from the nucleocapsid promoter do not modulate transcription from the minimal promoter in response to the RXR α -PPAR α nuclear receptors. Therefore, it appears that transcription from the nucleocapsid promoter can be modulated by binding of the RXR α -PPAR α nuclear receptors to an upstream regulatory element, probably the nuclear receptor binding site in the enhancer 1/X gene promoter region and nucleocapsid promoter HNF4 site 2, whereas HNF4 modulates transcription from the nucleocapsid promoter through nucleocapsid promoter HNF4 binding sites 1 and 2.

DISCUSSION

In this study, the influence of the nuclear receptors HNF4, RXR α , and PPAR α on HBV transcription was examined and

their roles in regulating the level of transcription from the nucleocapsid promoter were extensively characterized. HNF4 did not appear to influence the level of transcription from the enhancer 1/X gene promoter, whereas it modestly increased transcription from the major surface antigen promoter (two-fold) and the nucleocapsid promoter (threefold) in the context of the complete HBV genome (Table 1). The large surface antigen promoter was activated to the largest extent (ninefold) by HNF4. It was surprising that HNF4 did not activate the enhancer 1/X gene promoter, considering that there is a DR1 nuclear receptor binding site located in the HBV enhancer 1 region which binds HNF4 (17) and can activate transcription in an HNF4-dependent manner from a minimal promoter (Fig. 9). Despite this observation, it is apparent that HNF4 represents a liver-enriched transcription factor involved in the coordinate regulation of at least three of the four HBV promoters. The RXR α and PPAR α nuclear receptors increase the level of transcription from the nucleocapsid promoter and the large surface antigen promoter approximately fourfold in the context of the HBV genome, whereas they have less effect on the level of transcription from the major surface antigen promoter and the enhancer 1/X gene promoter, approximately 1.4- and 2.1-fold, respectively (Table 2). Based on these observations, it is likely that the RXR α and PPAR α nuclear receptors contribute to both the liver-specific and coordinate regulation of HBV transcription during viral infection.

The role of these transcription factors in controlling the level of transcription from the nucleocapsid promoter was characterized in detail. DNase I and gel retardation analyses established that two HNF4 binding sites were located in the nucleocapsid promoter spanning the regions from -132 to -102

FIG. 11. Clustered point mutational analysis of the nucleocapsid promoter. Relative activities of the clustered point mutation constructs in HepG2.1 cells in the absence (–R) or presence (+R) of exogenously expressed RXR α plus PPAR α polypeptides by using the expression vectors pRS plus pCMV and pRS-hRXR α plus pCMVPPAR α -G, respectively, are indicated. The relative activities of nucleocapsid promoter constructs were examined in the absence (–L) or presence (+L) of retinoic acid plus clofibrate as indicated. The activities were reported relative to the activity of the full-length promoter construct, CpLUC, in the absence of exogenously expressed nuclear receptor polypeptides and ligands. The standard deviation of the mean is shown in parentheses. The fold induction is the ratio of the relative activity in the presence of nuclear receptors and ligands to the relative activity in the absence of nuclear receptors and ligands. The internal control used to correct for transfection efficiencies was pCMV β . The diagrammatic representation of the construct CpLUC is as shown in Fig. 8. The horizontal lines indicate the HBV sequences present in the CpLUC plasmids. The HBV sequences deleted from the various plasmids are designated by nucleotide coordinates. The locations of the M1 and M2 mutations are indicated by solid boxes.

(HNF4 site 1) and –34 to –5 (HNF4 site 2) (Fig. 1). The primary transcription factor binding the HNF4 site 1 was HNF4, and it appears that additional members of the nuclear receptor superfamily including COUPTF1, ARP1, RXR α , and PPAR α do not interact to a large extent with this regulatory element (Fig. 5). In contrast, it appears that HNF4, COUPTF1, ARP1, RXR α , and PPAR α and possibly additional nuclear receptors interact with the nucleocapsid promoter HNF4 site 2 present within the CpFL regulatory sequence element.

The functional significance of these observations was investigated by transient-transfection analysis in the human dedifferentiated hepatoma cell line, HepG2.1. The two HNF4 sites in the nucleocapsid promoter can both independently mediate HNF4-dependent activation of transcription from the nucleocapsid promoter. However, it appears possible that factors present in HepG2.1 cells bind to HNF4 site 2 and reduce the ability of HNF4 to activate transcription from the nucleocapsid promoter as a result of HNF4 binding to HNF4 site 1. HNF4 binding to the DR1 nuclear receptor regulatory element in the enhancer 1/X gene promoter does not activate transcription from the nucleocapsid promoter (Fig. 8), despite the observation that this regulatory element is functional in the context of a minimal promoter (Fig. 9). This implies that HNF4 cannot act at a distance to activate transcription from the nucleocapsid promoter even though HNF4 activates transcription from the two HNF4 binding sites in the nucleocapsid promoter (Fig. 8).

The observation that the nucleocapsid promoter and the enhancer 1/X gene promoter contain DR1 nuclear receptor binding sites which bind multiple members of this superfamily of transcription factors including COUPTF1, ARP1, RXR α , and PPAR α , in addition to HNF4, suggested that these factors might modulate transcription from these promoters. It was demonstrated that the RXR α -PPAR α heterodimer modulated

transcription from these two promoters in a ligand-dependent manner (Table 2; Fig. 10). Mutational analysis of the two HNF4 binding sites in the nucleocapsid promoter in the context of the complete HBV genome suggested that the nuclear receptor binding site in the enhancer 1/X gene promoter could mediate the observed RXR α /PPAR α -dependent increase in transcription from the nucleocapsid promoter. However, HNF4 was unable to activate transcription from the nucleocapsid promoter containing mutations in HNF4 sites 1 and 2 despite being able to interact functionally with the enhancer 1/X gene promoter regulatory element. In addition, it was shown that RXR α -PPAR α can activate transcription from the nucleocapsid promoter by interacting with HNF4 site 2, and it is possible that the level of activation is reduced by a transcription factor present in HepG2.1 cells which binds to the HNF4 site 1. This situation is the opposite of that observed for HNF4-mediated transcriptional transactivation from the nucleocapsid promoter. It suggests that the level of transcription from the nucleocapsid promoter may be determined by both positive and negative interactions between different members of the nuclear receptor superfamily bound to the single HNF4 site located in the enhancer 1/X gene promoter region and the two HNF4 sites located in the nucleocapsid promoter. The possible role that the ARP1 transcription factor might play in regulating the level of expression from the nucleocapsid promoter was examined in transient-transfection analysis in HepG2.1 cells, but it does not appear to influence promoter activity at a detectable level (53).

The observation that RXR α and PPAR α influence the level of transcription from the nucleocapsid promoter suggests that modulating the effective concentrations of their ligands in the liver might alter the abundance of pregenomic RNA during a viral infection and consequently alter the level of HBV replication. Consequently, agonists and antagonists of the RXR α

FIG. 12. Functional analysis of the HBV nucleocapsid promoter HNF4 binding sites. The constructs examined contain the CpFL (nucleocapsid promoter HNF4 site 2), CpHNF4 (nucleocapsid promoter HNF4 site 1), XpHNF4 (enhancer 1/X gene promoter HNF4 site), PS1pHNF1 (large surface antigen promoter HNF1 site), and CpB (nucleocapsid promoter Sp1 site 2) double-stranded oligonucleotides cloned into the minimal promoter construct, pHBVTATALUC (see the insert). The number of copies of the oligonucleotide in the construct is shown in parentheses in the construct name. The relative activities of the constructs in HepG2.1 cells were examined in the absence (-R) or presence (+R) of exogenously expressed RXR α plus PPAR α polypeptides by using the expression vectors pRS plus pCMV and pRS-hRXR α plus pCMVPPAR α -G, respectively. The relative activities of the minimal promoter constructs were examined in the absence (-L) or presence (+L) of retinoic acid plus clofibrate, which is designated as having a relative activity of 1.0. The standard deviation of the mean is indicated by an error bar. The internal control used to correct for transfection efficiencies was pCMV β .

and PPAR α nuclear receptors might be developed as useful therapeutic agents to modulate the natural course of an HBV infection. In this context, it has recently been reported that specific mutations in the HNF4 site 2 DR1 recognition element, primarily an A-to-T change at nucleotide coordinate 1764 and a G-to-A change at nucleotide coordinate 1766 (Fig. 1), have been found in mutant viral genomes present in viremic patients who either have seroconverted from HBeAg positive to anti-HBe positive status or have reduced levels of HBeAg in serum (31, 42, 43, 67). It has been assumed that these mutations result in the loss or reduction of synthesis of the precore RNA encoding HBeAg whereas pregenomic RNA synthesis persists. This suggests that binding of members of the nuclear receptor superfamily of transcription factors to HNF4 site 2 might influence the relative abundances of the precore and pregenomic RNAs synthesized from the nucleocapsid promoter. Consequently, it is possible that modulation of the specific nuclear receptor occupying HNF4 site 2 in the nucleocapsid promoter will influence the relative abundances of these two 3.5-kb transcripts and therefore the level of HBeAg expression. Understanding the possible role that nuclear receptors, particularly ligand-dependent nuclear receptors, might play in transcription start site selection might suggest approaches to modulating the HBeAg status of HBV-infected individuals which may have useful therapeutic implications.

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