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Retinoid X Receptor α Transactivates the Hepatitis B Virus Enhancer 1 Element by Forming a Heterodimeric Complex with the Peroxisome Proliferator-Activated Receptor

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The hepatitis B virus enhancer 1 contains a retinoic acid responsive element (RARE). We have previously demonstrated that retinoid X receptor α (RXRα) transactivates enhancer 1 by binding to the RARE. The present study has revealed that a heterodimeric complex composed of RXRα and peroxisome proliferator-activated receptor (PPAR) interacts with the hepatitis B virus RARE. Transient transfection studies, in conjunction with in vitro DNA binding data, support the hypothesis that the RXRα-PPAR heterodimer transactivates enhancer 1.

Human hepatitis B virus (HBV) infectivity is characterized by a marked hepatotropism. The pathological manifestations of HBV infection include cirrhosis of the liver, which can lead to liver failure and death (11). Furthermore, epidemiological studies demonstrate a strong association between hepatocellular carcinoma and chronic HBV infection (4, 40). HBV contains a partially double-stranded, circular DNA genome that is approximately 3,200 nucleotides in length (Fig. 1A). Our laboratory (9, 22, 25) and others (3) have shown that the enhancer 1 element, located at nucleotides 966 to 1308 (HBV subtype adw), facilitates transcriptional activation from the promoter elements of HBV genes. Numerous studies have demonstrated that enhancer 1 functions in a liver-specific manner (3, 9, 20–22, 25, 35, 37, 41). Moreover, enhancer 1 activity appears to be governed by *trans*-acting cellular factors, which interact with specific sites on the enhancer (6, 25, 34, 35, 41). For example, the liver-enriched factors CCAAT/enhancer-binding protein (C/EBP) (15, 41) and retinoid X receptor α (RXRα) (17, 23) have been shown to transactivate enhancer 1. The RXRα binding site overlaps the FPV and FPIII sequence motifs (Fig. 1B), which are recognized by the hepatitis B enhancer-binding liver factor HBLF (23, 35, 41) and EF-C/RFX-1 (34, 38), respectively. The functional importance of this central region of the enhancer has been established by genetic studies (17, 20, 23, 41). Functional analyses have further suggested that enhancer 1 activity is promoted by cooperative interactions between enhancer binding factors (14, 15, 17). Therefore, a functional repertoire of ubiquitous and liver-enriched proteins may associate with each other to differentially regulate viral gene expression.

Recent work has indicated that liver-enriched members of the steroid-thyroid hormone receptor superfamily modulate the activity of the HBV enhancer 1 element. Nuclear receptors comprise a family of proteins that contain multiple conserved domains that dictate important structural and functional properties, including transcriptional activation and DNA/ligand binding (for reviews, see references 19 and 43). These receptors have been shown to activate transcription in both ligand-dependent (1, 5, 32) and ligand-independent (31, 36) manners. Moreover, nuclear receptors exert diverse effects upon cell differentiation, morphogenesis, and carcinogenesis (2, 10, 12, 39). Known members of this superfamily include retinoic acid receptor α (RARα), RARβ, and RARγ; RXRα, RXRβ, and RXRγ; peroxisome proliferator-activated receptor (PPAR); hepatocyte nuclear factor-4 (HNF-4); chicken ovalbumin upstream promoter transcription factor (COUP-TF); and receptors for thyroid hormone, estrogen, glucocorticoid, progesterone, vitamin D, androgen, and mineralocorticoid (19, 43). Recent studies have revealed that the liver-enriched nuclear receptors RXRα (17, 23) and HNF-4 (17) transactivate the HBV enhancer 1 by binding to the same site (Fig. 1B). COUP-TF appears to antagonize transcriptional activation by competing with RXRα and HNF-4 for binding to the same site on enhancer 1 (17). These findings suggest that RXRα is capable of regulating HBV gene expression by engaging in cooperative interactions with other nuclear receptors. While RXRα appears to be capable of functioning as a homodimer upon induction by 9-*cis*-retinoic acid (29, 46), it has been proposed that RXRα predominantly functions as a heterodimeric partner, or auxiliary protein, for other nuclear receptors (8, 27, 45).

RXRα has been shown to form a functional heterodimeric complex with PPAR (18, 28). PPAR is a liver-enriched nuclear receptor that has been implicated in the transcriptional regulation of genes encoding enzymes that are required for the peroxisomal β-oxidation of fatty acids (16, 24, 42, 44). Since the retinoic acid responsive element (RARE) on the HBV enhancer 1 (Fig. 1B) conforms with the direct repeat (DR) motif that is responsive to the RXRα-PPAR heterodimer (DR1) (18, 28), we have investigated the possibility that the RXRα-PPAR heterodimer transactivates enhancer 1. Therefore, the present study addresses the hypothesis that an RXRα-PPAR-mediated regulatory pathway(s) contributes to the liver-specific activation of HBV gene expression.

RXRα forms a stable complex with PPAR in the absence of DNA (28). Therefore, we initially utilized a protein-protein interaction assay to establish that RXRα and PPAR expressed in our laboratory were capable of interacting with each other in solution. Figure 2A shows in vitro-translated proteins that were used throughout this study. Equivalent amounts of in

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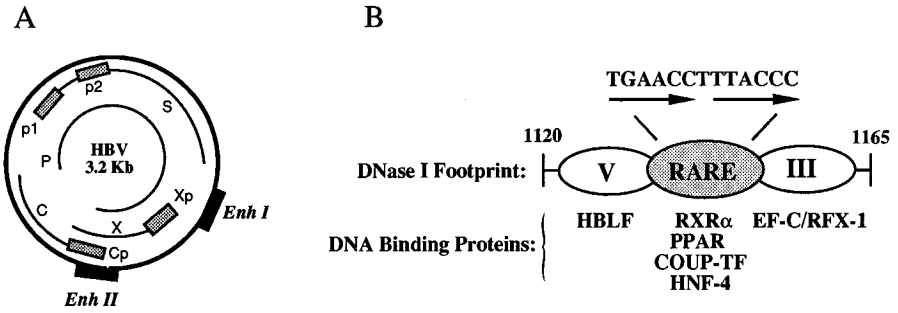

FIG. 1. (A) Genomic organization of HBV. S, C, P, and X represent the genes for the hepatitis B surface antigen, core/e antigen, polymerase, and X proteins, respectively. p1, p2, Cp, and Xp represent promoter regions of the corresponding distal genes. Enhancer elements 1 and 2 are designated Enh I and Enh II, respectively. (B) Protein binding sites on the FPV-FPIII domain of enhancer 1. The sequence of the HBV RARE DR is indicated. Nucleotide locations on enhancer 1 are shown.

vitro-translated ^{35}S-labeled proteins were incubated for 30 min at 25°C with an affinity matrix consisting of purified glutathione *S*-transferase (GST) or GST-RXRα fusion protein immobilized onto glutathione-Sepharose beads. Proteins that specifically bound to the affinity matrix are shown in Fig. 2B. In vitro-translated luciferase, which served as a negative control, did not interact with either affinity matrix (lanes 1 and 4). PPAR specifically interacted with GST-RXRα (lane 5, band a) yet did not adhere to GST (lane 2). As an additional control, we analyzed COUP-TF for its ability to interact with RXRα. Previous work has shown that COUP-TF heterodimerizes with RXRα (26) in the presence of DNA. We observed that

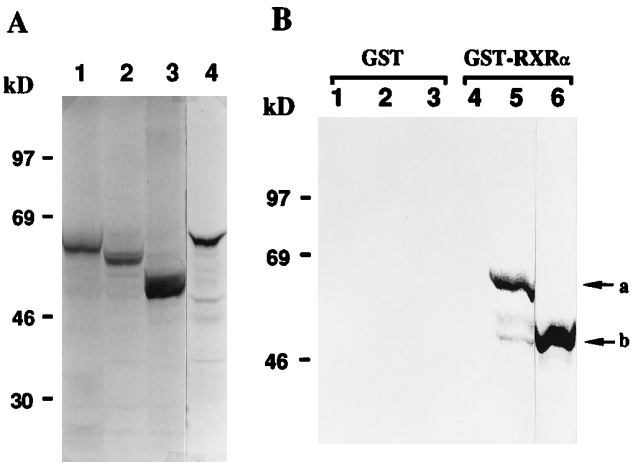

FIG. 2. RXRα and PPAR form a stable complex in the absence of DNA. (A) ^{35}S-labeled in vitro-translated RXRα (lane 1), PPAR (lane 2), COUP-TF (lane 3), and luciferase (lane 4) were resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (10% acrylamide), subjected to autoradiography, and quantitated by scanning densitometry. The migration positions of the following ^{35}S-labeled protein standards are indicated at the left: phosphorylase *b*, 97 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; and carbonic anhydrase, 30 kDa. PPAR and RXRα eukaryotic expression plasmids were prepared by cloning the respective cDNA into pGEM-3. PPAR, RXRα, COUP-TF, and luciferase expression plasmids were utilized for in vitro transcription and translation (TNT coupled transcription-translation system; Promega); in vitro translation was carried out in the presence or absence of 10 μCi of [^{35}S]methionine per ml for 90 min at 30°C. (B) GST-RXRα interacts with PPAR and COUP-TF in solution. GST and GST-RXRα (full length) were prepared as previously described (23). In vitro-translated proteins were incubated with either a GST or GST-RXRα affinity matrix as indicated. Proteins bound to the affinity matrix were extensively washed in the presence of 1% Triton X-100, boiled for 5 min in the presence of SDS loading buffer, and then resolved on an SDS-polyacrylamide gel (10% acrylamide), which was subsequently dried and analyzed with a PhosphorImager (Molecular Dynamics). Lanes 1 and 4, luciferase; lanes 2 and 5, PPAR; lanes 3 and 6, COUP-TF; band a, PPAR; band b, COUP-TF.

COUP-TF specifically binds to RXRα in the absence of DNA (lane 6, band b). Therefore, COUP-TF may preclude RXRα from transactivating the HBV enhancer 1 (17) by sequestering RXRα in a heterodimeric complex.

We proceeded to investigate whether the RXRα-PPAR heterodimer interacts with the HBV RARE. Protein-DNA binding was analyzed by the electrophoretic mobility shift assay. The following oligonucleotides, which span the RXRα binding site on enhancer 1, were used throughout this study: (i) wild type, 5′-GATCACAGTACATGAACCTTTACCCCGTT GCTC-3′ (nucleotides 1131 to 1159 of HBV subtype adw), and (ii) point mutant containing a G→A transition (23), 5′-GATC ACAGTACATAAACCTTTACCCCGTTGCTC-3′. As shown in Fig. 3A, GST-RXRα (lane 2) and GST-PPAR (lane 4) fusion proteins bound independently to the HBV RARE. However, we were unable to demonstrate the formation of a functional RXRα-PPAR heterodimer using the GST fusion proteins (data not shown). Therefore, as an alternative approach to examine this issue, the electrophoretic mobility shift assay was carried out using nonradioactive in vitro-translated proteins (Fig. 3B). It was intriguing to discover that in vitro-translated RXRα (lane 2) and PPAR (lane 3) did not exhibit binding activity. This finding suggests that these proteins are not capable of binding to the HBV RARE as native homodimers. Therefore, while GST-RXRα has been shown to specifically interact with the HBV RARE by DNase I footprinting analysis (23), the presence of the GST moiety on both receptors may impede the heterodimerization of GST-RXRα and GST-PPAR. A specific protein-DNA complex was observed only in the presence of in vitro-translated RXRα and PPAR (lane 4). To further examine the nature of this complex, samples were analyzed in the presence of polyclonal antibodies directed against RXRα (αRXRα), PPAR (αPPAR), and HNF-4 (αHNF-4). As shown in Fig. 3B, the specific complex was completely abrogated in the presence of αPPAR (lane 5) and αRXRα (lane 6). These results support the postulate that PPAR and RXRα are present in the specific complex observed in lane 4. A supershifted complex was observed in the presence of αRXRα (lane 6), which indicates the presence of an antibody-stabilized complex containing RXRα. The lack of a supershifted complex in the presence of αPPAR (lane 5) suggests that this antibody destabilizes the specific complex because of the presence of PPAR, which is consistent with previous observations (18). Furthermore, while an unrelated antibody, αHNF-4, had a destabilizing effect upon the nonspecific protein-DNA complex, this antibody did not affect the RXRα-PPAR-containing complex (lane 7). To further examine whether PPAR is present in this complex, a DNA competition experiment was carried out using nonradioactive com-

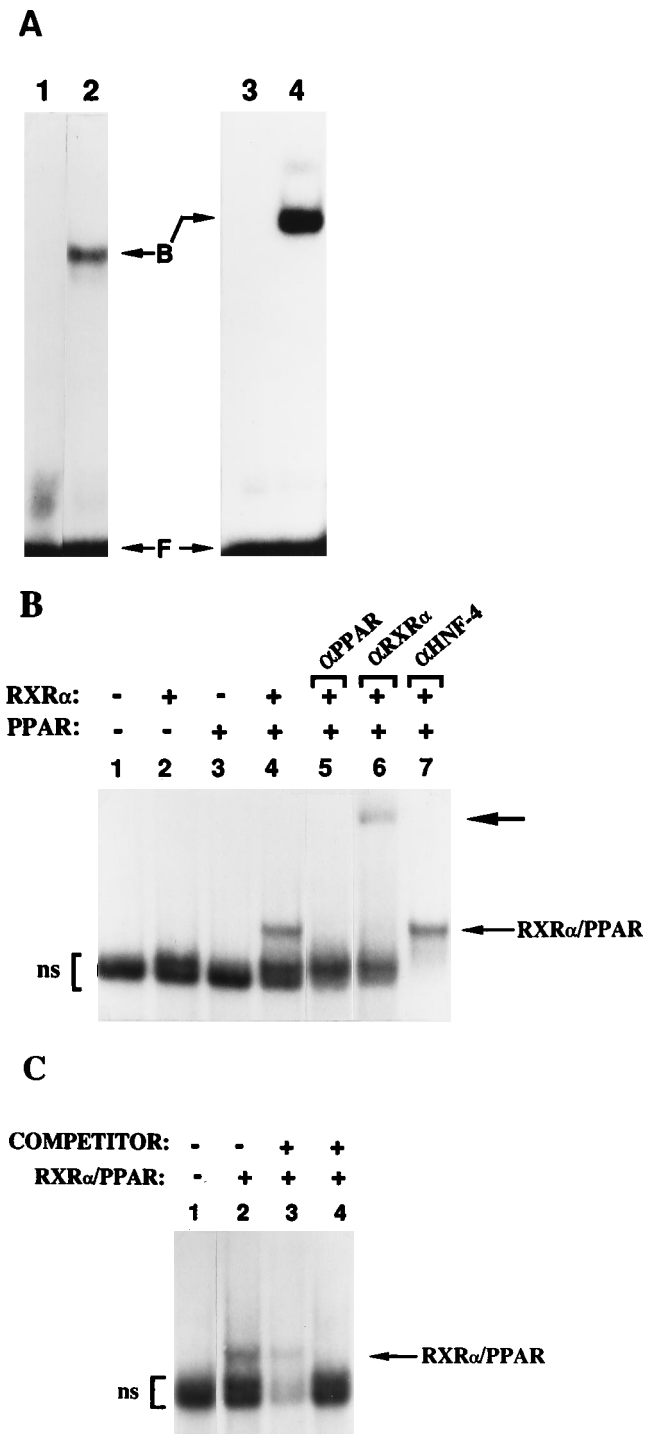

FIG. 3. The RXRα-PPAR heterodimeric complex interacts with the HBV RARE on enhancer 1. The electrophoretic mobility shift assay was carried out essentially as described elsewhere (23). The wild-type oligonucleotide, which contains the HBV RARE, was end labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. Following the DNA-protein binding reaction, certain samples were further incubated for 10 min at 4°C in the presence of polyclonal antibody. For DNA competition analysis, samples were incubated in the presence or absence of nonradioactive competitor DNA (50 ng). Following electrophoresis of 5% native polyacrylamide gels at 250 V and 4°C, the gels were dried and exposed to X-ray film at −70°C. (A) An expression plasmid encoding GST-PPAR was prepared by subcloning the full-length cDNA for PPAR (derived from pRSPPAR) into pGEX-3X (Promega). Samples were analyzed in the presence of no protein (lanes 1 and 3), GST-RXRα (lane 2), or GST-PPAR (lane 4). F, free probe; B, bound complex of protein-DNA. (B) Samples were analyzed in the presence (+) or absence (−) of nonradioactive in vitro-translated proteins (2 μl of programmed rabbit reticulocyte lysate for each protein), as indicated above each lane. Samples were normalized for total protein with unprogrammed rabbit reticulocyte lysate. Lanes 5 to 7 contain samples that were analyzed in the presence of the indicated polyclonal antibody. The small and large arrows indicate the migration positions of the RXRα-PPAR heterodimeric complex and the supershifted complex, respectively. Free probe is not shown. ns, nonspecific protein-DNA complex observed in the presence of unprogrammed rabbit reticulocyte lysate (lane 1). (C) Samples were analyzed in the presence or absence (lanes 1 and 2, respectively) of RXRα-PPAR (2 μl of the respective programmed lysate). Lanes 3 and 4 contain samples that were incubated in the presence of HBV (point mutant) and acyl coenzyme A oxidase oligonucleotide (50 ng in each case), respectively.

petitors (Fig. 3C). As a negative control, the mutated HBV oligonucleotide described above was shown to markedly reduce the nonspecific complex yet only partially reduced the specific complex (compare lanes 2 and 3). This result conforms with that of previous work (23), which detected weak binding of RXRα to full-length enhancer 1 containing the same point mutation. Accordingly, partial competition by the mutated HBV oligonucleotide is most likely due to a low-affinity interaction with RXRα. An oligonucleotide containing a PPAR-responsive element, which exists within the promoter region of the acyl coenzyme A oxidase gene (42), completely abrogated the specific complex (lane 4). This finding further supports the conclusion that PPAR is present in the specific complex. Taken together, the results shown in Fig. 3 are consistent with the hypothesis that an RXRα-PPAR heterodimeric complex interacts with the HBV RARE.

Transient transfection studies were then carried out to address the functional significance of the observations described above. Figure 4A shows the reporter plasmids that were used for this analysis. In the presence of their respective ligands, RXRα and PPAR were found to independently activate luciferase expression from the wild-type RARE (pGRSLuc) approximately eight- and sixfold, respectively (Fig. 4B). In addition, there was no induction observed with RXRα and PPAR in the absence of added all-*trans*-retinoic acid (23) and ciprofibrate (23a), respectively. Since the in vitro DNA binding data suggested that RXRα and PPAR do not bind to the HBV RARE as homodimers (Fig. 3B, C), RXRα and PPAR most likely activated the HBV RARE by forming heterodimeric complexes with endogenous, liver-enriched nuclear receptors that are present in Huh-7 cells. Furthermore, because the cell medium was carbon stripped of endogenous steroids, the sixfold induction by PPAR in the presence of ciprofibrate demonstrates that the HBV RARE is activated by a PPAR-mediated regulatory pathway. When RXRα and PPAR were transfected together in the presence of their respective ligands, luciferase expression from pGRSLuc was activated approximately 17-fold over that in the control sample. This level of enhancement, over that observed with either receptor alone, suggests that RXRα and PPAR transactivate the HBV RARE by forming a functional heterodimeric complex. Moreover, these receptors were unable to activate pGR′SLuc either alone or in combination, which further supports the hypothesis that RXRα and PPAR transactivate HBV gene expression by binding to the RARE on enhancer 1. While RXRα and PPAR may activate the HBV RARE by interacting with other nuclear receptors, such as RAR, HNF-4, thyroid hormone, and/or vitamin D receptors (7, 17, 27, 45), our results exclusively support the hypothesis that RXRα and PPAR interact with each other to mediate enhancer 1 activation.

Our present data support the notion that the RXRα-PPAR

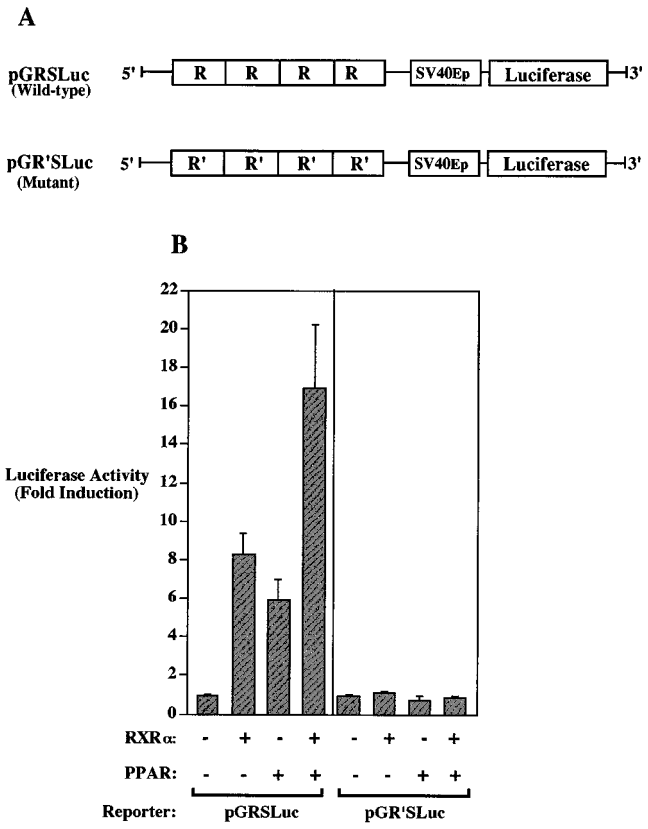

FIG. 4. Functional analysis of the HBV RARE. (A) The wild-type (R) and mutated (R′) oligonucleotides described in the text were used to generate clones containing multimerized binding sites. Double-stranded R and R′ were independently multimerized in the presence of DNA ligase. Multimers containing four copies were isolated and cloned into pGEM-3 to generate pGRXRα (wild type) and pGRXRα′ (point mutant). DNA sequencing (Sequenase 1.0; U.S. Biochemicals) was performed to confirm the orientation and copy number of the multimerized region. A DNA fragment containing the simian virus 40 early promoter (SV40Ep) upstream of the firefly luciferase gene was excised from plasmid pSLuc2 (33) and subcloned into pGRXRα and pGRXRα′ to generate the reporter plasmids pGRSLuc (wild type) and pGR′SLuc (point mutant), respectively. (B) Transient transfection assays were performed using the human hepatoma cell line Huh-7 (23). Cells were plated at a density of $\sim 5 \times 10^5$ cells per 60-mm dish and were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum (carbon stripped to remove endogenous steroids) and penicillin (75 U/ml)-streptomycin (50 U/ml) at 37°C. Cell monolayers (~50% confluent) were transfected by the calcium phosphate precipitation method using 2 μg of reporter plasmid in the presence (+) or absence (−) of 0.1 μg of RXRα and/or PPAR expression plasmid. All plates incubated with RXRα- and PPAR-expressing plasmids were treated with all-*trans*-retinoic acid and ciprofibrate, respectively. Four hours posttransfection, plates were furnished with fresh medium containing 0.1% dimethyl sulfoxide, 10^{-5} M all-*trans*-retinoic acid, or 0.5 mM ciprofibrate. The cells were further incubated for 40 h, harvested, and then analyzed for luciferase expression (13). The relevant protein expression and reporter plasmids are indicated below the graph. Luciferase activity is expressed as fold induction over the baseline level of activity observed in the absence of ligand induction and receptor expression plasmids. A β-galactosidase expression plasmid, pCH110, was utilized as an internal control for transfection efficiency. The means and standard deviations were derived from at least two independent experiments.

heterodimeric complex is a physiological activator of the HBV RARE. This hypothesis is consistent with the tissue-specific distribution of RXRα and PPAR, which are both predominantly expressed in the liver (24, 30). Although previous work has shown that RXRα (46) and PPAR (42) are capable of independently interacting with their respective responsive elements, this study and results of others (18, 28) have shown that these receptors preferentially function as a heterodimeric complex. While the HBV RARE exhibits the DR1 consensus that is recognized by the RXRα-PPAR heterodimer (28), the nucleotide sequence of the HBV RARE is divergent at several nucleotide positions. These differences may account for the preferential binding of the RXRα-PPAR heterodimer to the HBV RARE. Moreover, the divergent nature of the HBV RARE may dictate a multitude of interactions between RXRα, PPAR, and/or other known or unknown members of the nuclear receptor superfamily. Furthermore, the proximity of the HBV RARE to other protein binding sites (Fig. 1B) suggests that members of the nuclear receptor superfamily, including RXRα and PPAR, facilitate enhancer 1 transactivation by engaging in a cooperative interaction(s) with another cellular factor(s). Future studies that address this hypothesis may ultimately increase our understanding of the relationship between HBV gene expression and the pathological manifestations of chronic HBV infection.

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