Retinoid X receptor RXR α binds to and trans-activates the hepatitis B virus enhancer

(retinoic acid induction/liver-specific enhancer)

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ABSTRACT A retinoid X receptor (RXR) response element was located within the functionally defined hepatitis B virus (HBV) enhancer element. A short segment of the enhancer that contains this region has been shown with genetic analysis to play a key role in the regulation of enhancer function and to represent a major determinant of liver-specific activity. Both the full-length protein and the DNA-binding domain of the liver-specific receptor RXR α bound to the putative retinoic acid response element in the HBV enhancer. *In vivo*, an HBV enhancer-reporter gene construct responds to induction with retinoic acid when cotransfected with an RXR α expression vector. A single-base transition (G $\rightarrow$ A) in the HBV retinoic acid response element leads to a dramatic reduction both in the *in vitro* binding activity of RXR α and the *in vivo* activity of the HBV enhancer. Thus, retinoic acid and the RXR α are implicated as being significant determinants in the liver-specific regulation of HBV gene expression and the resultant disease pathogenesis.

Retinoic acid (RA) plays a crucial role in vertebrate development and cell differentiation. These effects of RA are achieved by binding of RA to RA receptors (RARs) and transforming these receptors to active transcriptional factors (1, 2). Two families of RARs have been characterized. The RAR gene family consists of three well-characterized genes that includes RAR α , β , and γ (ref. 3 and the references therein). These RARs share homology with the superfamily of steroid and thyroid hormone receptors and bind all-*trans*-RA with high affinity. The second class of nuclear receptor, referred to as retinoid X receptor (RXR), includes three members of RXR family, RXR α , β , and γ (4–7). These receptors respond to much higher concentrations of all-*trans*-RA. Recently, however, a retinol metabolite called 9-*cis*-RA has been identified that binds with 40-fold greater affinity and, thus, may represent the physiological ligand for RXRs (8). Although both RARs and RXR α are effective activators of gene expression, RXR α differs substantially in primary structure and ligand specificity from the family of RARs (4). Expression of RXR α is highly enriched in the liver. Different tissue distribution and timing of the expression of these receptors ensure regulation of specific set of genes during cell growth and differentiation.

In addition to acting as a nuclear factor for transcription in the presence of its ligand, RXR α can also function as an auxiliary protein for RARs, thyroid hormones, and vitamin D $_3$ receptor (7, 9, 10). In doing so, RXR forms heteromeric complexes with the other receptors, which allows the dimer to bind more effectively and contribute to a large repertoire of transcription factors with distinct functional properties (7, 9, 10).

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Several genes that are activated in response to RA have been identified. Some of these genes include those encoding alcohol dehydrogenase, ApolA, and CREBII (ref. 11 and the references therein; refs. 12–14). A RA response element (RARE) is located in the enhancer/promoter regions of these genes. One feature of the RARE is that they all contain direct repeats of the half-site, TGACCT, or a closely related sequence in various combinations and spacing (11, 15).

The human hepatitis B virus (HBV) is a partially double-stranded DNA virus (16). Expression of the HBV genes appears to be regulated by transcriptional control elements (promoters/enhancers) that display preference for liver cells (17–19). This expression is facilitated by the interaction between trans-acting cellular factors and the upstream regulatory sequences in HBV genome (Fig. 1). Two enhancer elements (I and II) have been identified in the HBV (20, 21). The enhancer I maps at nucleotide (nt) positions 966–1308. DNase I protection assay has revealed several regions of DNA-protein interactions in the enhancer (Fig. 1B). Binding of several factors has been characterized (17, 22–25). These include both ubiquitous transcription factors, such as NF-1, EF-C, and CREB/ATF proteins; and liver-specific factors, including C/EBP, hepatitis B liver factor, and RXR α (ref. 25 and this paper). By mutational analysis, Trujillo *et al.* (25) identified the minimal sequences that retain the major function of the enhancer. This region consists of sequences that produced the DNase I-protected footprints (FPs) III and V. Ubiquitous transcriptional factor EF-C has been shown to bind the FPIII region (24). An unidentified liver-specific factor has been shown to bind the FPV region (25). In this analysis we have located a RARE sequence motif that resides between FPIII and FPV regions. Because liver has been shown to be enriched in RXR α , we reasoned that this interaction may be one of the key factors responsible in regulating the HBV gene expression. This study was undertaken to demonstrate the functional interaction of RXR α with the enhancer element. Using gel-mobility-shift and DNase I-protection assay, we demonstrate here the interaction of RXR α with the HBV enhancer *in vitro*. We further show that RXR α stimulates the viral enhancer function linked to a reporter gene *in vivo*. Mutations in the RARE of the HBV enhancer result in a dramatic decrease in the binding of RXR α *in vitro*, and in cotransfection assays the induction of the enhancer activity by RA is obliterated.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis. Both the eukaryotic (pRXR α) (4) and prokaryotic (pGEX-2T-RXR α) (14) expression vectors for RXR were from R. Evans, Salk

Abbreviations: HBV, hepatitis B virus; RLNE, rat liver nuclear extract; FP, DNase I-protected footprint; RA, retinoic acid; RARE, RA response element; nt, nucleotide(s); RXR, retinoid X receptor; RAR, RA receptor; FPIII and FPV, footprint III and V, respectively; RXR α _{DBD}, DNA-binding domain of RXR α .

FIG. 1. (A) Genomic organization of the HBV. S, C, Pol, and X represent genes for hepatitis B surface antigen, core/e antigen, polymerase, and X proteins. P1, P2, Cp, and Xp represent promoter regions of the corresponding distal genes. The enhancer elements are indicated as Enh I and Enh II. Kb, kilobases. (B) Proteins binding sites of the enhancer I. The putative binding sites of RXR α and hepatitis B liver factor are shown.

Institute (La Jolla, CA). DNA-binding domain of the RXR α contained within nt 237–776 (*Sac* I–*Bam*HI) was excised from phRXR α (4) corresponding to amino acid residue 56–234 and cloned in pGEX-2T (26) to produce the plasmid pGTXD (Fig. 2A). Site-directed mutations were generated with the Altered Sites Mutagenesis system (Promega). The two mutants, termed EnR' and En5', represent mutations in the RARE motif and the FPV region, respectively. Dideoxynucleotide chain-termination sequencing analysis was done to confirm the presence of mutations. The wild-type and the mutated enhancers (966–1308 nt) were then placed in front of the simian virus 40 early promoter in pSluc2 (27). The construction of the plasmids containing the HBV enhancer sequences linked to luciferase reporter gene has been described (25).

Expression of the DNA-Binding Domain of RXR α . *Escherichia coli* JM101 was transformed with plasmid pGTXD. Cells were grown under conditions of isopropyl β -D-thiogalactoside induction. Bacterial lysates of both the control plasmid expressing only the glutathione S-transferase (pGEX-2T) and the hybrid fusion protein containing RXR α DNA-binding domain (RXR α _{DBD}) (pGTXD) were purified on glutathione-agarose resin (26). Protein concentrations were determined by a Bio-Rad assay. Full-length bacterially expressed RXR α (14) was prepared by the similar method (26).

Gel-Mobility-Shift Assay. HBV enhancer fragment, *Hpa* I–*Hpa* II (966–1308 nt), or the oligonucleotide containing the putative RXR α -binding sequences was radiolabeled at the 5' end with [γ -³²P]ATP by T4 polynucleotide kinase. About 15,000 cpm (³²P) of purified probes were incubated with purified fusion-protein GT–RXR α _{DBD} or glutathione S-transferase (G) for 30 min on ice. The incubation was carried out in a 15- μ l vol with 20 mM Tris (pH 7.5)/100 mM KCl/10 mM MgCl₂/1 mM EDTA/1 mM dithiothreitol/poly(dI-dC) at 0.1 mg/ml. The reaction mixtures were resolved on a 5% polyacrylamide gel in 0.5 $\times$ Tris/borate/EDTA (TBE; 1 $\times$ TBE is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) buffer. Gel electrophoresis was done at 200 V at room temperature in 0.5 $\times$ TBE buffer. The dried gels were exposed against x-ray films at –70°C.

DNase I-Protection Assay. The DNase I-protection analysis was done as described (28). Rat liver nuclear extracts (RLNEs) were prepared from fresh rat liver tissues as described by Johnson *et al.* (29). The reaction products were subjected to electrophoresis through 8% urea-polyacrylamide gels, dried, and autoradiographed with an intensifying screen and Kodak x-ray film.

Cell Transfection and RA Induction. Human hepatoma cell line Huh-7 (30) was used in the transfection assays. Cells were transiently cotransfected with 5 μ g of plasmid that contains the enhancer I (wild-type or mutants) linked to the luciferase reporter gene and 0.5 μ g of vector phRXR α by the calcium phosphate precipitation method. Expression vector phRXR α contains the human RXR α gene under the control of Rous sarcoma virus long terminal repeat (4). The β -galactosidase plasmid pCH110 was used as an internal control to

normalize transfection values. Transfected cells were incubated for 12 hr and were further incubated for another 24 hr with or without all-*trans*-RA (Eastman Kodak). Cells were harvested, and the protein extracts were prepared for the β -galactosidase assay and normalized for subsequent luciferase assay (31).

RESULTS

Binding of RXR α to the HBV Enhancer. We investigated the binding of RXR α to the HBV enhancer I element. Because the DNA-binding domains of transcriptional factors can bind to their target sequence (32, 33), we expressed the DNA-binding domain of RXR α as a glutathione S-transferase–RXR α hybrid gene product (Fig. 2). The soluble protein GT–RXR α _{DBD} was purified by binding to a glutathione-agarose affinity resin. SDS/PAGE analysis revealed the elution of a fusion protein with the predicted molecular mass of 48 kDa (Fig. 2B). Full-length RXR α was also purified by using a similar expression system (14, 26). Both full-length RXR α and RXR α _{DBD} were used in all the DNA-binding

FIG. 2. (A) Construction of bacterial expression plasmid pGTXD. Schematic representation of plasmid pGTXD containing the glutathione S-transferase (GT) and RXR α _{DBD} fusion is shown. Expression of the hybrid gene is under the control of *tac* promoter. (B) Expression of GT–RXR α _{DBD} in *E. coli*. Bacterial lysates expressing control plasmid pGEX-2T (lanes 2–4) and pGTXD (lanes 5–7) are fractionated through an SDS/10% polyacrylamide gel and stained with Coomassie blue. Lanes: 2 and 5, uninduced lysates; 3 and 6, isopropyl β -D-thiogalactoside-induced lysates; 4 and 7, lysates purified on glutathione-agarose affinity resin (26). Molecular mass markers (in kDa) are shown in lane 1.

studies. The DNA probe used in these studies consists of the RARE oligonucleotide. The putative RARE motif is located at nt 1138 in the enhancer element. Results of gel-mobility-shift assays are described in Fig. 3A. The binding was seen with both the full-length and DNA-binding domain of the RXR α (lanes 3 and 6). The sequences contained in the *Sac*I–*Bam*HI fragment cloned in pGTXD plasmid include 178 amino acids of RXR α , of which only 76 amino acids have been defined as representing the DNA-binding domain. It is likely that the pGTXD plasmid may represent other functional domains of RXR α (see Fig. 4). The binding of RXR α appears heat-labile (lanes 5 and 8). Specificity of binding was confirmed by competition with unlabeled RARE oligonucleotide (lanes 4 and 7). The enhancer fragment containing the RARE motif was also used in gel-shift assays and produced similar results (see Fig. 5, lane 2). The binding activity of RAR or RXR *in vitro* is independent of RA, as has been noted (14, 33).

To map the binding site for RXR α , DNase I-protection analysis (28) was done with the DNA-binding domain of RXR α using the whole enhancer fragment (966–1308 nt) (Fig.

FIG. 3. Binding of purified RXR α . (A) Gel-mobility-shift analysis of binding of purified fusion proteins (RXR α DBD and full-length RXR α) to HBV enhancer. 5'-End-labeled oligonucleotide containing the RARE motif of enhancer I (nt 1138–1167) was incubated with 5 μ l of RXR α DBD, lanes 3–5, and with full-length RXR α , lanes 6–8. G, glutathione S-transferase; R, unlabeled RARE oligonucleotide; H, heat treated. Arrows: B, bound; F, free. (B) DNase I-protection analysis of the enhancer. DNA-binding domains of RXR α (10 ng/ μ l) and RLNE (10 μ g/ μ l) were incubated with the radiolabeled *Hpa*I–*Hpa*II (966–1308 nt) enhancer fragment and subjected to limited DNase I digestion. RLNE produces the typical pattern of DNase I-protected regions as described (25). Amounts of proteins are indicated in μ l above lanes. The RXR footprint is marked at left. RXR α , RXR α DBD; H, heat treated; G+A, sequencing ladder.

3B). Although RLNE generated a typical pattern of DNA–protein interactions, RXR α fusion protein produced a distinct footprint overlapping FPIII and part of FPV. This footprint includes the sequence 5'-TGAACCTTAACCC-3'. These sequences match the common RARE sequence motif DR-1 with a space of 1 nt (14, 34).

As has been noted (33, 34), binding of RARs was enhanced in the presence of cellular extracts. We observed similar effect on the binding of RXR α in the presence of RLNEs. A high affinity of interaction occurred with both the full-length RXR α fusion protein (Fig. 4, lane 10), and the DNA-binding domain of RXR α (lane 5) with RLNE. Nonspecific proteins, such as bovine serum albumin, did not enhance RXR α binding (data not shown). These complexes maintained the same pattern of migration as RXR α alone (lanes 3 and 8). The identity of these factor(s) contributing to a higher affinity of binding is currently unknown.

Binding of RXR α to the Enhancer Mutants. To confirm the specificity of RXR α interactions with the enhancer element, mutations were introduced at two sites in the enhancer element by site-directed mutagenesis. The mutation in the RARE motif (EnR') was a single-base transition from guanine to adenine, resulting in the conversion of one of the repeat sequence 5'-TGAACC-3' to 5'-TAAACC-3'.

The second mutation was introduced in the AGTA repeat sequence of the FPV. These mutations destroyed the repeat sequence (En5'). The rationale of generating this mutation was to determine the influence of RXR α interaction on binding of factor(s) at the FPV. The close proximity of AGTA repeats to the RARE motif and lack of identity of protein(s) binding at FPV also raised the question of whether RXR α was the factor or one of the factors responsible for producing FPV. Both mutations (EnR' and En5') were analyzed with a gel-shift-mobility assay for binding to the DNA-binding domain of RXR α . The mutation in RARE (EnR') abolished the binding (Fig. 5, lane 4). However, the mutations in the FPV region (En5') did not affect the binding of RXR α (lane 3). This result suggested that proteins binding at the FPV region and those at the RARE motif represent independent interactions with their cognate sequence.

Activation of Enhancer Function by RXR α . To establish the functional relevance of the RXR binding, we investigated the

FIG. 4. Enhancement of RXR α binding with nuclear extracts. Gel-mobility-shift analysis was done by using 5'-end-radiolabeled RARE oligonucleotide as a probe with purified RXR α DBD (lanes 2, 3, and 5) and full-length RXR α proteins (lanes 7, 8, and 10). Concentrations of RXR α DBD (10 ng/ μ l), RXR α (10 ng/ μ l), and RLNE (3 μ g/ μ l) are indicated in μ l. Arrows: B, bound; F, free.

FIG. 5. Gel-mobility-shift analysis of HBV enhancer mutants. 32 P-labeled wild-type enhancer (lane 2), En5' (lane 3), and EnR' (lane 4) fragments were incubated with 100 ng of RXR α DBD (lane 2-4) and analyzed. Lane 1, enhancer probe alone (*Hpa* I-*Hpa* II, 966-1308 nt).

possibility of trans-activation/repression of the HBV enhancer function by the receptor in response to RA. Cotransfection assays were done with plasmid pHnLuc containing the HBV enhancer/X gene promoter (966-1374 nt) linked to the luciferase reporter gene and the RXR α expression plasmid pHXR α (Fig. 6A). A human hepatoma cell line, Huh-7 (30), which is negative for HBV sequences, was used in all transfection studies. Cotransfection with the expression vector pHXR α alone had no effect on the basal enhancer activity (data not shown), but the induction of luciferase activity in response to RA was seen. RA concentration of 4×10^{-6} M induced the highest level of luciferase expression under control of the HBV enhancer (Fig. 6A).

We further examined the enhancer mutants in transfection assays. Wild-type and mutant enhancers (966-1308 nt) were cloned in pSluc2 in which the luciferase gene is under the transcriptional control of the simian virus 40 early promoter. pEnR'Sluc contains a mutation in RARE and pEn5'Sluc in the region of FPV in which the direct repeats (AGTA) have been altered. In the plasmid pENSluc, luciferase gene is under the control of simian virus 40 early promoter and wild-type HBV enhancer. Huh-7 cells were transfected with these constructs, together with the RXR α expression plasmid (Fig. 6B). To our knowledge the simian virus 40 early promoter does not contain RARE and, thus, is not stimulated by RA. Although expression of the wild-type construct

pENSluc is stimulated by RA and the receptor by >5-fold, the enhancer mutants do not respond to induction by RA. It was further noted that the luciferase expression under the transcriptional control of the mutant enhancers was reduced. This suggests that RA response sequences and those in the FPV region (perhaps the AGTA repeats) play an important role in the overall enhancer function. These results show that RXR α is involved in regulating the enhancer activity *in vivo* and provide evidence that RXR α may mediate the apparent stimulation by binding to the RARE motif in the enhancer element.

DISCUSSION

The purpose of this study was to determine whether HBV gene expression is regulated by retinoids. The rationale for this inquiry is derived from genetic studies with the enhancer in which a segment of ≈ 50 base pairs was shown sufficient for supporting enhancer activity. Two enhancer elements of HBV have been implicated directly in controlling the levels of viral gene expression (17, 18, 21). In this study, we demonstrate the interaction of RXR α with the RARE motif centrally located in the enhancer element I. The sequence represents an RXR α direct repeats with a spacing of 1 nt. This arrangement and spacing of sequences are consistent with the DR-1 common motif found in the RXR element motif of several gene promoters and enhancers (14, 34). Half-site spacing preference may be used in regulating gene expression (11, 15, 34). A point mutation (EnR') in the HBV RARE motif abolishes binding of RXR α to the enhancer *in vitro*. In support of this observation, results from cotransfection studies suggest that while the HBV enhancer responds to stimulation by RXR α and RA, the EnR' mutation causes a significant decrease in enhancer activity and responsiveness to RA induction. An independent point mutation in the FPV region (En5') also affects enhancer function, lending support to the earlier observation regarding the importance of 50 base pairs in sustaining the major function of the enhancer (25). However, DNase I-protection analysis of the En5' mutant enhancer will be necessary to confirm this observation. The tissue-specific distribution of RXR α is consistent with the role it plays in regulating HBV and other liver-specific gene expression. Because HBV infects hepatocytes and efficient gene expression is primarily restricted to the liver, it is logical to assume that the machinery of liver gene expression is effectively used by HBV. In doing so, the enhancer has evolved consolidated sequence motifs to which several liver-specific and ubiquitous transcriptional factors bind to facil-

FIG. 6. (A) RA induction of the HBV enhancer. Five micrograms of pHnLuc and 0.5 μ g of pHXR α (4) were used to transfect Huh-7 cells in the presence of increased concentrations of all-*trans*-RA. (B) RA induction of HBV enhancer mutants. Luciferase expression was assayed 48 hr after transfection, according to de Wet *et al.* (31). Five micrograms of pENSluc (wild-type enhancer), pENR'Sluc (RARE mutant), pEN5'Sluc (FPV mutant), and pSluc2 was used to transfect Huh-7 cells, together with 0.5 μ g of pHXR α . Twelve hours after transfection, cultures were induced with either 4×10^{-6} M RA or ethanol (negative control). Each point represents an average of two sets of experiments.

itate HBV gene expression. The state of hepatocytic growth and the availability of factors may dictate efficiency of enhancer/promoter function. Of interest in this respect is the RXR α (high in liver) that binds the viral enhancer and contributes to the hepatotropism exhibited by the virus. Although RXR α response element has been localized in the control elements of a few genes preferentially expressed in liver, its presence in the HBV enhancer reinforces its specific role in regulating gene expression in liver. If RA is a major signal transducer of liver gene expression, the RARE motif becomes an important component of the viral enhancer after induction by RA. In this role, RA may act as a potent inducer of chronic viral infection and the disease pathogenesis of liver associated with it, including hepatocellular carcinoma. Interestingly, a site of HBV genome integration has been located in the RAR β gene (35).

It has been shown that RXR α interacts with RARs, thyroid receptors (TRs), and vitamin D₃ receptors and activates the transcriptions of the target genes (7, 10, 11). Recent studies (34) also show that RXR and COUP-TF can form heterodimer and modulate RA signaling by repressing RXR α function. Although our studies are based on interaction of RXR α with the HBV enhancer, actual receptors that bind to the enhancer *in vivo* could be either an RXR α homodimer or heterodimers of RXR α and other relevant receptors. Our studies, in which a high-affinity complex appears in the presence of nuclear extracts (Fig. 4), strongly supports the hypothesis that RXR α is interacting with cellular factor(s) during formation of this complex. Also likely are the possibilities that small molecules (i.e., nucleotides, etc.) (36) or redox state (37) of RXR α may contribute to the high affinity of interaction without altering the migration pattern of the retarded complexes.

An important question is whether RA and RXR α can regulate HBV gene expression via their interaction with RARE motif located in the enhancer element. We and others have previously demonstrated that enhancer I regulates the various HBV promoters and thus controls overall viral gene expression (17–19).

Finally, it is clear that HBV gene expression is regulated by the availability of factors, the state of hepatocytic growth, differentiation, and the presence of cytokines and hormones. In this scenario, the signal transduction of RA by RXR α may play a crucial and decisive role in the infectious processes of viral hepatitis and liver oncogenesis.

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