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INDEPENDENT ACTIVATION OF HEPATITIS B VIRUS BIOSYNTHESIS BY RETINOIDS, PEROXISOME PROLIFERATORS AND BILE ACIDS

Running title: Transcriptional regulation of HBV replication

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ABSTRACT

In the human hepatoma cell line, HepG2, retinoic acid, clofibric acid and bile acid treatment can only modestly increase HBV biosynthesis. Utilizing the human embryonic kidney cell line, 293T, it was possible to demonstrate that the retinoid X receptor α (RXRα) plus ligand can support viral biosynthesis independently of additional nuclear receptors. In addition, RXRα/peroxisome proliferator-activated receptor α (PPARα) and RXRα/farnesoid X receptor α (FXRα) heterodimeric nuclear receptors can also mediate ligand dependent HBV transcription and replication when activated by clofibric acid and bile acid, respectively, independently of a requirement for the ligand dependent activation of RXRα. These observations indicate that there are at least three possible modes of ligand-mediated activation of HBV transcription and replication existing within hepatocytes suggesting multiple independent mechanisms control viral production in the liver of infected individuals.

INTRODUCTION

HBV infection is primarily restricted to hepatocytes in the liver. This restriction is believed to occur at two distinct levels (32). The receptor(s) involved in viral entry are presumably present only on hepatocytes and govern species specificity (11). In addition, viral biosynthesis is restricted in a tissue and cell-type specific manner because HBV transcription is dependent on liver-enriched transcription factors (39,40). A variety of nuclear receptors have been shown to regulate HBV pregenomic 3.5kb RNA synthesis and hence viral replication (28,29,33). Three of these nuclear receptors, RXR, PPAR and FXR, are ligand dependent transcription factors that are activated by retinoids, peroxisome proliferators and bile acids, respectively (23,25). Therefore it is apparent that the ligands for these nuclear receptors might be critical determinants of viral biosynthesis under both normal and pathophysiological conditions within the liver of infected individuals (12,34).

As the ligand-activated heterodimeric nuclear receptors, RXRα/PPARα and RXRα/FXRα, regulate HBV pregenomic RNA synthesis by the recruitment of coactivators, it was of interest to evaluate the relative contribution of the individual heterodimeric partners to the overall level of viral transcription and replication (28,29,44). Characterization of the relative role of individual polypeptides to the transcriptional activity of various heterodimeric nuclear receptors has been evaluated (9,10,37). This approach indicated that one partner might play a dominant role in controlling promoter activity depending on the nuclear receptors involved (9,10,37).

Therefore it was of interest to evaluate the effects of retinoids, peroxisome proliferators and bile acids, alone or in combination, on HBV transcription and replication.

In the current study, it is demonstrated that retinoids can activated HBV biosynthesis utilizing both RXRα containing homodimers and heterodimers. Alternatively HBV transcription and replication can be supported by RXRα/PPARα and RXRα/FXRα in the absence of retinoids when these heterodimeric transcription factors are activated by peroxisome proliferators and bile acids, respectively. Additionally, it appears that the activation of both heterodimeric partners does not dramatically enhance the level of HBV transcription and replication compared to the level of viral biosynthesis observed with a single nuclear receptor ligand. These observations suggest that a single ligand can efficiently activate the heterodimeric nuclear receptors governing HBV biosynthesis independently of the ligand binding status of its partner. This indicates that the ligand induced conformational changes occurring in one polypeptide that are necessary for coactivator recruitment can occur either independently of its heterodimer partner or the conformational change in the ligand bound polypeptide is concurrently induced in the heterodimeric partner without its requirement to bind ligand (37). If nuclear receptor antagonists are to be considered as potential antiviral agents for the treatment of chronic HBV infections, it will be critical to distinguish between these different mechanisms of action so appropriate therapeutic modalities might be considered.

MATERIALS AND METHODS

Plasmid constructions.

The HBV DNA (4.1kbp) construct that contains 1.3 copies of the HBV genome includes the viral sequence from nucleotide coordinates 1072 to 3182 plus 1 to 1990 (40). The pRS-hRXRα, pCMV-rFXRα, pCMVPPARα-G, pCMX-hRARα, pCMX-mPXR.1, pCMX-mLXRα and pCMX-hCAR vectors express RXRα, FXRα, PPARα-G, retinoic acid receptor α (RARα), pregnane X receptor (PXR.1), liver (oxysterol) X receptor α (LXRα) and constitutive androstane receptor (CAR) polypeptides from the human RXRα , rat FXRα, mouse PPARα-G, human RARα, mouse PXR.1, mouse LXRα and human CAR cDNAs, respectively, using the Rous sarcoma virus LTR (pRS) or the CMV immediate-early promoter (pCMV and pCMX) (6,13,15,22,24,27,42). The PPARα-G polypeptide contains a mutation in the PPARα cDNA changing Glu^{282} to Gly that may decrease the affinity of the receptor for the endogenous ligand. Consequently, this mutation increases the peroxisome proliferator-dependent (i.e. clofibric acid-dependent) activation of transcription from a peroxisome proliferator response element (PPRE) containing promoter (27).

Cells and transfections.

The human hepatoma HepG2 cell line and human embryonic kidney 293T cell line were grown in RPMI-1640 medium and 10% fetal bovine serum at 37°C in 5% CO_2/air. Transfections for viral RNA and DNA analysis were performed as previously described (26) using 10 cm plates, containing approximately 1 X 10^6 cells. DNA and RNA isolation was performed 3 days post transfection. The transfected DNA mixture

was composed of 5 μg of HBV DNA (4.1kbp) plus 1.5 to 6.5 μg of the nuclear receptor expression vectors, pRS-hRXRα, pCMV-rFXRα, pCMVPPARα-G, pCMX-hRARα, pCMX-mPXR.1, pCMX-mLXRα and pCMX-hCAR (6,13,15,22,24,27,42). Controls were derived from cells transfected with HBV DNA and the expression vectors lacking a nuclear receptor cDNA insert (31). All-*trans* retinoic acid, 9-*cis* retinoic acid, chenodeoxycholic acid and clofibric acid at 0.05-10 μM, 0.05-10 μM, 100 μM and 1 mM, respectively, were used to activate the nuclear receptors, RXRα, FXRα and PPARα (13,20,40).

Characterization of HBV transcripts and viral replication intermediates.

Transfected cells from a single plate were divided equally and used for the preparation of total cellular RNA and viral DNA replication intermediates as described previously (38) with minor modifications. RNA (Northern) and DNA (Southern) filter hybridization analysis were performed using 10 μg of total cellular RNA and 30 μl of viral DNA replication intermediates, respectively, as described (36). Filter hybridization analyses were quantified by phosphorimaging using a Packard Cyclone Storage Phosphor System.

RESULTS

Retinoids, peroxisome proliferators and bile acids modulate HBV biosynthesis in human hepatoma HepG2 cells.

Transfection of the HBV DNA (4.1kbp) construct into HepG2 cells supports HBV transcription and replication (Fig. 1A and B, lane 1). Treatment of HepG2 cells with retinoic acid, clofibric acid and chendeoxycholic acid modestly enhanced the level of HBV biosynthesis (Fig. 1A and B, lanes 2, 5 and 8) suggesting that these hepatoma cells express RXR (and/or RAR), PPAR and FXR that are capable of being activated in the presence of exogenously added ligand. Exogenous expression of RXRα, PPARα and FXRα in the absence of added ligands had a limited effect on HBV biosynthesis (Fig. 1A and B, lane 3, 6 and 9). Exogenous expression of RXRα, PPARα and FXRα in the presence of added ligands resulted in a similar level of viral replication as seen with ligand alone (Fig. 1B, lane 4, 7 and 10). Although all of these effects in HepG2 cells are relatively modest, they indicate that HepG2 cells contain excess inactive RXR (and/or RAR), PPAR and FXR that is capable of enhancing HBV biosynthesis if activated by the appropriate ligand. In addition, it appears that ligand-activated PPAR or FXR might be able to enhance HBV biosynthesis in the absence of ligand activated RXR. However due to the modest nature of the effects in HepG2 cells (Fig. 1C), it is not possible to conclusively determine the relative roles of the various nuclear receptors and their ligands in HBV biosynthesis without examining their effects in a more tractable system.

Ligand-activated RXRα supports HBV biosynthesis in human embryonic kidney 293T cells.

Transfection of the HBV DNA (4.1kbp) construct with the RXRα expression vector into 293T cells fails to support HBV transcription and replication in the absence of ligand (Fig. 2A and C, lanes 1 and 2). Activation of RXRα by all-*trans* retinoic acid, presumably by its isomerization to 9-*cis* retinoic acid (13,20), or 9-*cis* retinoic acid treatment of the 293T cells resulted in a dose-dependent induction of both HBV RNA and DNA synthesis (Fig. 2). The half maximal induction of HBV biosynthesis was observed at approximately 1.6 μM 9-*cis* retinoic acid and 3.6 μM all-*trans* retinoic acid (Fig. 2). The relatively high concentrations of retinoids required to activate RXRα suggest that RAR, which can be activated by both all-*trans* and 9-*cis* retinoic acid (5,8,16,17), is probably not the RXRα heterodimer partner involved in mediating HBV transcription and replication in 293T cells. Additionally the requirement for high concentrations of 9-*cis* retinoic acid, the natural ligand for RXRα (13,20), suggests that the intracellular concentration of this retinoic acid isomer must be relatively low presumably due to the activity of endogenous isomerases within the 293T cells (see Discussion for details). Therefore these observations suggest that RXRα either activates HBV biosynthesis as a homodimer or as a heterodimer with PPARα or FXRα although the latter suggestion seems less likely given the embryonic origin of the 293T cell line (18).

Selective effects of specific nuclear receptors on ligand-activated RXRα-mediated HBV biosynthesis in human embryonic kidney 293T cells.

Unlike the situation in HepG2 cells, transfection of the HBV DNA (4.1kbp) construct into 293T cells in the presence of retinoid acid fails to support HBV transcription and replication (Fig. 3A and B, lanes 1 and 2). In addition, the exogenous expression of RXRα also failed to support HBV biosynthesis in the absence of retinoic

163 acid in 293T cells (Fig. 3A and B, lane 3). However, the exogenous expression of RXRα
164 in the presence of retinoic acid is sufficient to support robust HBV transcription and
165 replication (Fig. 3A and B, lane 4). To address the potential role additional nuclear
166 receptors might have in governing HBV biosynthesis in 293T cells, several potential
167 heterodimer partners were over-expressed in the presence of a constant amount of co-
168 expressed RXRα polypeptide (Fig. 3A and B, lanes 5-11). Over-expression of RXRα by
169 transfecting approximately 4-fold more of the RXRα expression vector did not greatly
170 modulate HBV biosynthesis (Fig. 3A and B, lanes 4 and 5). Comparable over-expression
171 of FXRα, PPARα, LXRα and CAR did not greatly modulate RXRα-mediated HBV
172 biosynthesis (Fig. 3A and B, lanes 7, 8, 10 and 11) suggesting that in the absence of their
173 ligands these nuclear receptors did not affect viral transcription and replication (Fig. 3C).
174 Interesting, over-expression of RARα inhibited RXRα-mediated HBV biosynthesis (Fig.
175 3A and B, lane 6). This observation strongly suggests that the RXRα/RARα heterodimer
176 does not activate HBV biosynthesis and therefore it appears likely that RXRα can
177 directly activate HBV transcription and replication in the absence of any additional ligand
178 activated nuclear receptors. The mechanism(s) of inhibition of HBV biosynthesis by
179 RARα and PXR is unclear (Fig. 3A and B, lanes 6 and 9). It is possible that these
180 nuclear receptors might shift the cellular equilibrium from RXRα homodimers capable of
181 activating HBV transcription to RXRα/RARα or RXRα/PXR heterodimers that are
182 incapable of binding to the nucleocapsid promoter to activate HBV transcription. This
183 equilibrium shift would reduce HBV biosynthesis but then it is difficult to rationalize
184 why LXRα and CAR would not affect viral RNA and DNA synthesis in a similar
185 manner. In the case of the RXRα/RARα heterodimer, it is likely that binding to the

direct repeat 1 (DR1) sequence comprising two copies of the AGGTCA related sequence separated by a single nucleotide within the HBV nucleocapsid promoter mediates corepressor recruitment and hence transcriptional repression (16,17,31). In contrast, RXR/PXR does not bind to DR1 sequences (4,15) but might sequester coactivators such as peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) that can activate nuclear receptor-mediated HBV biosynthesis (3,21,28,29).

Independent activation of HBV biosynthesis by retinoids, peroxisome proliferators and bile acids in human embryonic kidney 293T cells.

Previously, it has been shown that when both nuclear receptor partners are ligand-activated RXRα plus PPARα and RXRα plus FXRα can support HBV biosynthesis in nonhepatoma cells (28,29,33,40). The relative role of the individual heterodimer polypeptides in nuclear receptor-dependent activation of HBV biosynthesis has not been evaluated. In 293T cells, transfection of the HBV DNA (4.1kbp) construct in the presence of retinoid acid fails to support HBV transcription and replication (Fig. 4A and B, lane 16). In addition, the exogenous expression of RXRα also failed to support HBV biosynthesis in the absence of retinoic acid (Fig. 4A and B, lane 17). However, the exogenous expression of RXRα in the presence of retinoic acid is sufficient to support robust HBV transcription and replication (Fig. 4A and B, lane 18). These observations suggest that an RXRα homodimer is sufficient to support HBV biosynthesis in 293T cells. However, it is also possible that RXRα may be forming heterodimers with an endogenously expressed nuclear receptor and this transcription factor complex is directing the expression of the HBV pregenomic 3.5kb RNA. To evaluate this possibility, the role of known functional RXRα partners was evaluated for their effects

on HBV biosynthesis in the presence or absence of activating ligands. FXRα, its ligand alone or both together failed to support robust HBV transcription or replication (Fig. 4A and B, lanes 2-4). RXRα plus FXRα in the absence of ligands also failed to support robust HBV biosynthesis (Fig. 4A and B, lane 5). In contrast, RXRα plus FXRα in the presence of ligands supported robust HBV biosynthesis (Fig. 4A and B, lane 6-8). Activation of FXRα by its ligand, chendeoxycholic acid, appeared to be somewhat more potent than retinoic acid alone but the combination of both ligands was only somewhat greater than the effect observed with chendeoxycholic acid alone. Similar observations were apparent when PPARα and clofibric acid were evaluated with RXRα and retinoic acid (Fig. 4A and B, lane 9-15). Overall, these results indicate that ligand-activated RXRα can activate HBV biosynthesis in nonhepatoma cells. In the presence of FXRα or PPARα, RXRα/FXRα or RXRα/PPARα heterodimers can bind to the HBV nucleocapsid promoter and direct viral pregenomic RNA synthesis either through ligand-dependent activation of the common heterodimer partner, RXRα, or conversely through ligand-dependent activation of the unique heterodimer partner. HBV transcription does not appear to require the activation of both polypeptides in the heterodimer although activation of the unique partner or both partners appears to induce slightly more robust viral biosynthesis.

DISCUSSION

Nuclear receptors are a major determinant of HBV tropism because they contribute to the liver-specific expression of the viral 3.5kb pregenomic RNA (28,29,40). This HBV transcript encodes the viral polymerase and core polypeptides and is reverse transcribed into the 3.2kbp partially double-stranded genomic DNA present in the virion (41). Nuclear receptors are ligand-dependent transcription factors and hence their activities are governed by the availability of their specific ligand (23,25). Consequently, nuclear receptors represent potential targets for drug development because small molecular weight compounds can act as agonist or antagonists to modulate their activities and hence alter disease progression. Indeed, the fibrate class of compounds has been used to modulate PPARα activity and treat hypertriglyceridemia and hypercholesterolemia (43). Likewise, the thiazolidinedione class of compounds which act on PPARγ represent a group of anti-diabetic drugs and tamoxifen is an anti-estrogen compound used in the treatment of breast cancer (19,35). Therefore understanding the mechanism of action of ligands capable of modulating the activities of the nuclear receptors governing HBV biosynthesis may be informative in the development of strategies aimed at treating chronic HBV infections.

Initially the human hepatoma cell line, HepG2, was treated with retinoid, a peroxisome proliferator and a bile acid to examine the role of the nuclear receptors, RXR (and/or RAR), PPAR and FXR in the regulation of HBV transcription and replication (Fig. 1). Treatment with retinoic acid, clofibirc acid and chendeoxycholic acid enhanced HBV biosynthesis indicating that HepG2 cells express RXR (and/or RAR), PPAR and

FXR capable of being activated by the appropriate ligand. As PPARα and FXRα bind to their recognition sequences in the HBV nucleocapsid promoter as RXR/PPAR and RXR/FXR heterodimers (31,33,40), these observation suggest that HepG2 cells contain RXR capable of forming transcriptionally active complexes with their activated heterodimer partners. Consistent with this assumption is the observation that retinoic acid can activate HBV biosynthesis in HepG2 cells without the exogenous expression of any additional nuclear receptors. Therefore it appears that HepG2 cells contain inactive RXR which can be activated by relatively high concentrations of retinoic acid when it is converted into 9-*cis* retinoid acid, the natural ligand for RXR (13,20). Alternatively, the RXR homodimer may be capable of supporting HBV biosynthesis when activated by retinoids without the requirement for any interaction with additional nuclear receptors. However, due to the high constitutive level of HBV biosynthesis mediated by endogenous transcription factors and the relatively modest induction of viral RNA and DNA synthesis by ligand-activated nuclear receptors in HepG2 cells, a definitive characterization of the relative roles of ligands and their corresponding nuclear receptors in governing HBV biosynthesis was not possible in this system. To address this limitation, the nonhepatoma HBV replication system where viral transcription is completely dependent on the activation of exogenously expressed nuclear receptors by their cognate ligands was exploited (Fig. 2-4).

HBV transcription and replication in the human embryonic kidney cell line, 293T, required the ligand-dependent activation of exogenously expressed RXRα (Fig. 2). 9-*cis* retinoic acid and all-*trans* retinoic acid treatment of 293T cells displayed dose dependent increases in HBV RNA and DNA synthesis with half maximal levels being observed at a

concentration of approximately 1.6 μM and 3.6 μM, respectively. The modest preference of RXRα for its cognate ligand, 9-*cis* retinoic acid, and the relatively high concentrations of retinoids required for this level of viral biosynthesis were initially unexpected. In the monkey kidney cell line, CV-1, half maximal levels of transcription from RXR responsive promoters are observed at approximately 50 nM 9-*cis* retinoic acid and 2 μM all-*trans* retinoic acid (13,20). However the reason the 9-*cis* retinoic acid was relatively ineffective at activating HBV biosynthesis might be due to the 64 hour treatment with retinoids required for the evaluation of viral RNA and DNA production. It is possible that during this extended incubation period both the 9-*cis* retinoic acid and the all-*trans* retinoic acid are rapidly isomerized to a similar pool of retinoic acid isomers resulting in similar effective intracellular concentrations of the RXRα ligand, 9-*cis* retinoic acid (13,20). Consequently, there is only a minor shift in the dose response curves when HBV biosynthesis is evaluated and a relatively high concentration of retinoid is required because only a minor fraction is the appropriate isomer (13,20). Regardless of the reason for these observations, it is apparent that ligand-activated RXRα is directly activating viral transcription presumably by binding to the HBV nucleocapsid promoter DR1 sequence as a homodimer (31,33,40).

Previously, RXRα/PPARα and RXRα/FXRα heterodimers have been shown to activate HBV transcription and replication (14,28-31,33,40). The relative importance of the different polypeptides in these heterodimers and the role of their ligands in the activation of HBV biosynthesis have not been established. The observation that ligand-activated RXRα alone is sufficient to support HBV RNA and DNA synthesis raised the issue of the role of additional RXRα heterodimer partners in governing the level of HBV

297 biosynthesis. Initially, additional nuclear receptors capable of heterodimerizing with
298 RXRα were evaluated for their effects on retinoic acid-activated RXRα-mediated HBV
299 RNA and DNA synthesis (Fig. 3). Interestingly, FXRα, PPARα, LXRα and CAR failed
300 to modulate ligand-activated RXRα-mediated HBV transcription and replication (Fig. 3).
301 In contrast, RARα and PXR inhibited ligand-activated RXRα-mediated HBV
302 biosynthesis (Fig. 3). The observation that RARα inhibited retinoic acid-activated
303 RXRα-mediated HBV biosynthesis is consistent with the suggestion that RXRα/RARα
304 heterodimers activate transcription when bound to direct repeat 5 (DR5) transcriptional
305 regulatory elements but repress transcription when bound to DR1 transcriptional
306 regulatory elements (16,17). As the HBV nucleocapsid promoter contains a functional
307 DR1 transcriptional regulatory element that binds various nuclear receptors but lacks a
308 known functional DR5 transcriptional regulatory element (31,33,40), it appears that the
309 binding of an RXRα homodimer to this DR1 element activates viral transcription
310 whereas the binding of an RXRα/RARα heterodimer represses HBV biosynthesis (Fig.
311 3). The observation that the expression of CAR, which binds to DR5 transcriptional
312 regulatory elements as a RXR/CAR heterodimer (1,6), did not affect ligand-activated
313 RXRα-mediated HBV biosynthesis is also consistent with this suggestion (Fig. 3).
314 Together these observations suggest that enhanced RAR activity may inhibit HBV
315 biosynthesis under some conditions and therefore may have some therapeutic benefits for
316 chronic HBV carriers. Interestingly, the nuclear receptor PXR also inhibited retinoic
317 acid-activated RXRα-mediated HBV biosynthesis. PXR binds to direct repeat 3 (DR3),
318 DR4 and inverted repeat 6 (IR6) transcriptional regulatory elements as a RXR/PXR
319 heterodimer and is activated by various steroid hormones, xenobiotic drugs including

rifampicin and dietary compounds such as phytoestrogens (2,4,15). The absence of detectable PXR response elements within the HBV transcriptional regulatory sequence elements suggests PXR might inhibit ligand-activated RXRα-mediated HBV biosynthesis by sequestering RXRα as RXRα/PXR heterodimers apart from the viral nucleocapsid promoter. However, if PXR can inhibit HBV transcription in this manner it is unclear why LXRα and CAR cannot also prevent viral RNA synthesis by a similar mechanism. Alternatively, PXR might act in a manner similar to RAR or by preventing coactivator mediated activation of RXRα (3,16,17,21). Further analysis will be required to determine the mechanism of PXR-mediated inhibition of ligand-activated RXRα-mediated HBV biosynthesis but these observations suggest that activation of PXR by small molecular weight compounds might represent another possible approach for the treatment of chronic HBV infections.

The observation that FXRα and PPARα failed to enhance retinoic acid-activated RXRα-mediated HBV biosynthesis in the absence of their cognate ligands (Fig. 3) questioned the importance of their role in HBV biosynthesis. However in the absence of retinoic acid, bile acids and peroxisome proliferators activated HBV biosynthesis demonstrating that these ligands were critical determinants of viral pregenomic RNA synthesis as part of the RXRα/FXRα and RXRα/PPARα heterodimer complexes, respectively (Fig. 4). Inclusion of both ligands for either nuclear receptor heterodimer combination did not synergistically enhance HBV RNA and DNA synthesis suggesting that activation of any one partner in the heterodimer was sufficient to induce the majority of the potential transcriptional activity associated with the two nuclear receptor polypeptides. A further modest enhancement of transcriptional activity was apparent

when the second ligand was included but overall these results suggest that the activation of a single nuclear receptor by ligand binding is sufficient to support most of the observed RNA synthesis from the HBV nucleocapsid promoter. These observations suggest that either a single nuclear receptor within the heterodimer is capable of recruiting the majority of the transcriptional machinery necessary for maximal promoter activity or the activation of one partner by ligand binding leads to the activation of the other partner presumably by appropriate allosteric interactions (7,9,10,37).

The observation that peroxisome proliferators activate viral transcription and replication *in vivo* in the HBV transgenic mouse model of chronic infection demonstrate that activation of PPARα is functionally important in this animal system (12). Similarly, bile acids can activate HBV biosynthesis to a limited extent *in vivo* under certain circumstance (34). Together these findings suggest that ligand-activated nuclear receptor-mediated HBV biosynthesis may represent a proportion of the virus production occurring during natural infection whereas the remainder of the HBV RNA and DNA synthesis is probably mediated by orphan nuclear receptors (34) plus additional classes of liver-enriched and ubiquitous transcription factors (39). Consequently therapeutic approaches aimed at the reduction or elimination of HBV transcription will probably benefit from selective targeting of both ligand-dependent and ligand-independent modes of viral RNA synthesis if this step in the HBV life cycle is going to be efficiently inhibited.

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FIGURE LEGENDS

Figure 1. Effects of retinoids, peroxisome proliferators and bile acids on HBV biosynthesis in the human hepatoma cell line, HepG2. (A) RNA (Northern) filter hybridization analysis of HBV transcripts. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for RNA loading per lane. (B) DNA (Southern) filter hybridization analysis of HBV replication intermediates. HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single stranded DNA. Cells were transfected with the HBV DNA (4.1kbp) construct alone (lane 1) or the HBV DNA (4.1kbp) construct plus the RXRα (lanes 3 and 4), PPARα (lanes 6 and 7) and FXRα (lanes 9 and 10) expression vectors as indicated. In addition, cells were treated with 1 μM retinoic acid (RA; lanes 2 and 4), 1mM clofibric acid (CA; lanes 5 and 7) and 100mM chendeoxycholic acid (CDCA; lanes 8 and 10) to activate the RXRα, PPARα and FXRα nuclear receptors, respectively. (C) Quantitative analysis of the HBV 3.5-kb RNA and HBV DNA replication intermediates. The levels of the HBV 3.5-kb RNA and total HBV DNA replication intermediates are reported relative to the HBV DNA (4.1-kbp) construct in the absence of nuclear receptor expression or ligand (lane 1). The mean RNA and DNA levels plus standard deviations from four independent analyses are shown. The levels of the transcripts and replication intermediates in the nuclear receptor and/or ligand treated cells

which are statistically significantly higher than their levels in the corresponding untreated cells by a Student's t-test ($p<0.05$) are indicated with an asterisk (*).

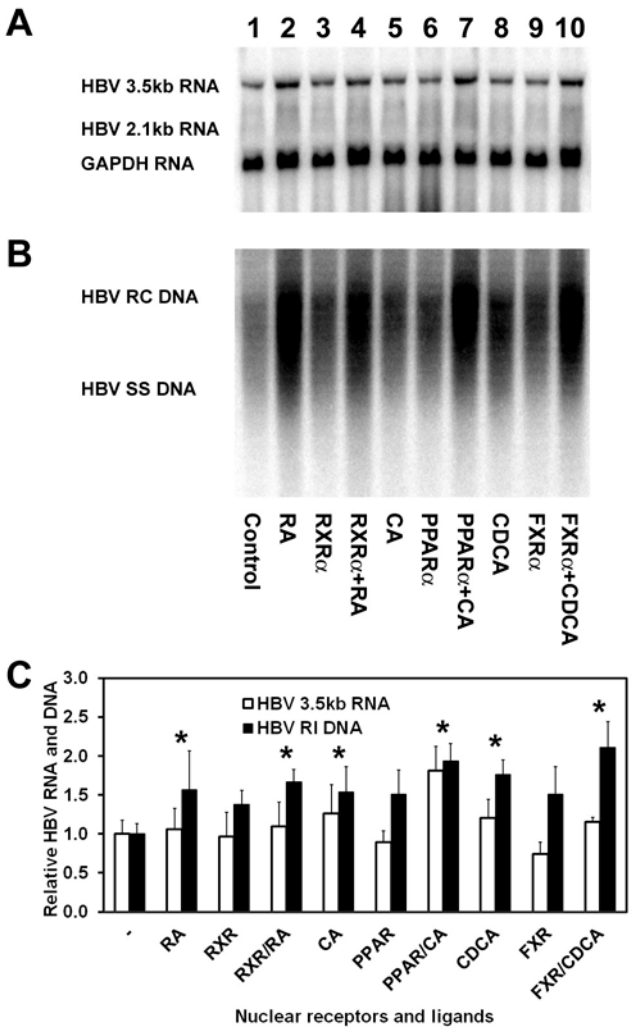
Figure 2. Effect of all-*trans* retinoic acid and 9-*cis* retinoic acid concentration on HBV biosynthesis in the human embryonic kidney cell line, 293T, expressing RXRα. Cells were transfected with the HBV DNA (4.1kbp) construct alone (lane 1) or the HBV DNA (4.1kbp) construct plus the RXRα expression vector (lanes 2-14) as indicated. Cells were treated with various concentrations of all-*trans* retinoic acid (0.05-10 μM RA; lanes 3-8) and 9-*cis* retinoic acid (0.05-10 μM 9-*cis* RA; lanes 9-14). (A) RNA (Northern) filter hybridization analysis of HBV transcripts. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for RNA loading per lane. (B) Quantitative analysis of the HBV 3.5-kb RNA from two independent experiments. Trend lines were calculated using GraphPad Prism 5 software to determine the sigmoidal dose response (variable slope) curve plus EC_{50} values. (C) DNA (Southern) filter hybridization analysis of HBV replication intermediates. HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single stranded DNA. (D) Quantitative analysis of the HBV replication intermediates from two independent experiments. Trend lines were calculated using GraphPad Prism 5 software to determine the sigmoidal dose response (variable slope) curve plus EC_{50} values.

Figure 3. Effects of nuclear receptor expression on RXRα-mediated HBV biosynthesis in the human embryonic kidney cell line, 293T. Cells were transfected with 5 μg of the HBV DNA (4.1kbp) construct alone (lanes 1 and 2) or 5 μg of the HBV DNA (4.1kbp) construct plus 1.5 μg of the RXRα expression vector (lanes 3-11) as indicated. In addition, cells were treated with 10 μM retinoic acid (RA; lanes 2 and 4-11) to activate RXRα. An additional 5 μg of nuclear receptor RXRα (lane 5), RARα (lane 6), FXRα (lane 7) PPARα, (lane 8), PXR (lane 9), LXRα, (lane 10) and CAR (lane 11) expression vectors were also included in the transfections as indicated. (A) RNA (Northern) filter hybridization analysis of HBV transcripts. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for RNA loading per lane. (B) DNA (Southern) filter hybridization analysis of HBV replication intermediates. HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single stranded DNA. (C) Quantitative analysis of the HBV 3.5-kb RNA and HBV DNA replication intermediates. The levels of the HBV 3.5-kb RNA and total HBV DNA replication intermediates are reported relative to the HBV DNA (4.1-kbp) construct in the presence of RXRα expression and 10 μM all-*trans* retinoic acid (lane 4), which are designated as having a relative activity of 1.0. The mean RNA and DNA levels plus standard deviations from two independent analyses are shown. The levels of the transcripts and replication intermediates which are statistically significantly lower than observed in the all-*trans* retinoic acid-

587 treated RXR-expressing cells by a Student's t-test ($p<0.05$) are indicated
588 with an asterisk (*).

589 Figure 4. Independent activation of HBV biosynthesis by retinoids, peroxisome
590 proliferators and bile acids in the human embryonic kidney cell line, 293T.
591 Cells were transfected with the HBV DNA (4.1kbp) construct alone (lanes
592 1, 2, 9 and 16), the HBV DNA (4.1kbp) construct plus the RXRα
593 expression vector (lanes 5-8, 12-15, 17 and 18), the HBV DNA (4.1kbp)
594 construct plus the FXRα expression vector (lanes 3-8), or the HBV DNA
595 (4.1kbp) construct plus the PPARα expression vector (lanes 10-15) as
596 indicated. In addition, cells were treated with 1 μM retinoic acid (RA;
597 lanes 7-8, 14-16 and 18) to activate RXRα, 100 μM chendeoxycholic acid
598 (CDCA; lanes 2, 4, 6 and 8) to activate FXRα and 1 mM clofibric acid
599 (CA; lanes 9, 11, 13 and 15) to activate PPARα as indicated. (A) RNA
600 (Northern) filter hybridization analysis of HBV transcripts. The
601 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used
602 as an internal control for RNA loading per lane. (B) DNA (Southern)
603 filter hybridization analysis of HBV replication intermediates. HBV RC
604 DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single stranded
605 DNA. (C) Quantitative analysis of the HBV 3.5-kb RNA and HBV DNA
606 replication intermediates. The levels of the HBV 3.5-kb RNA and total
607 HBV DNA replication intermediates are reported relative to the HBV
608 DNA (4.1-kbp) construct in the presence of RXRα expression and 1 μM
609 all-*trans* retinoic acid (lane 18). The mean RNA and DNA levels plus

standard deviations from two independent analyses are shown. The levels of the transcripts and replication intermediates in the ligand treated cells which are statistically significantly higher than their levels in the corresponding untreated cells by a Student's t-test ($p<0.05$) are indicated with an asterisk (*).



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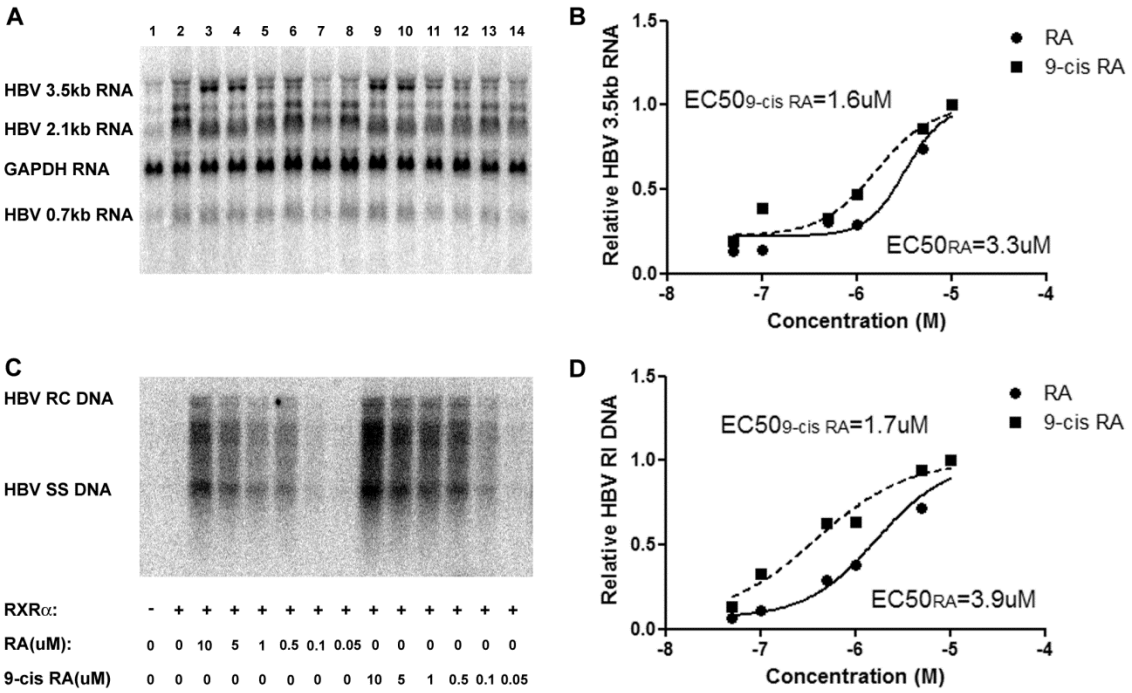

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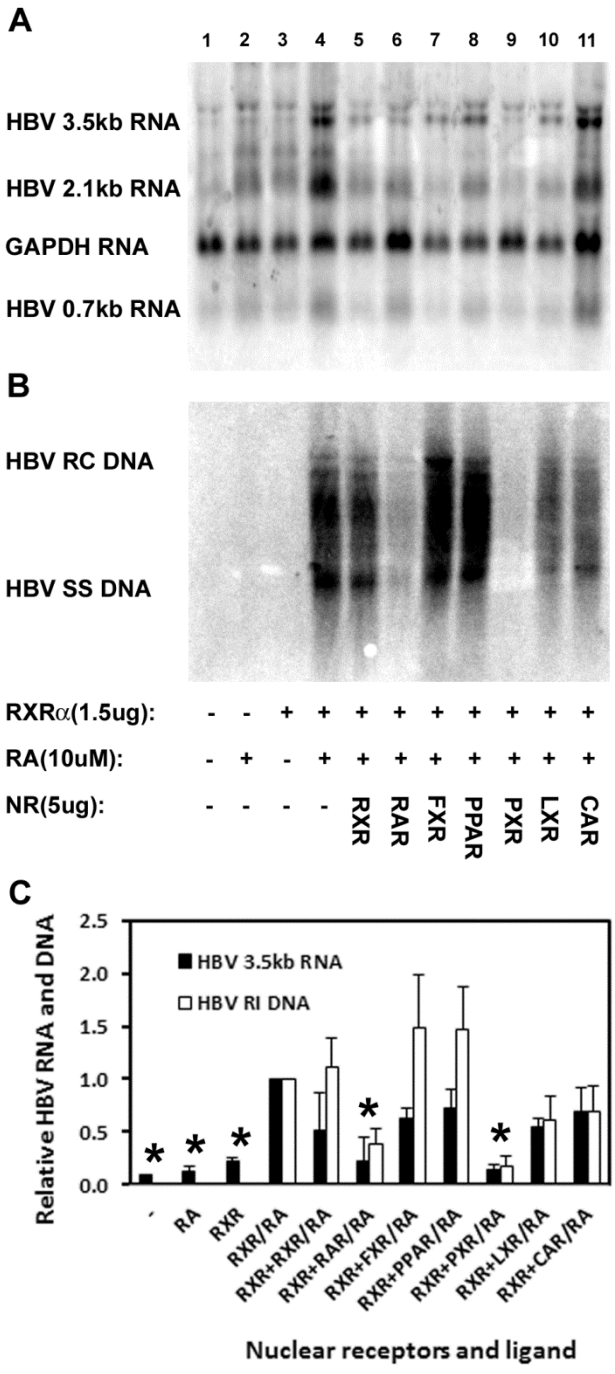

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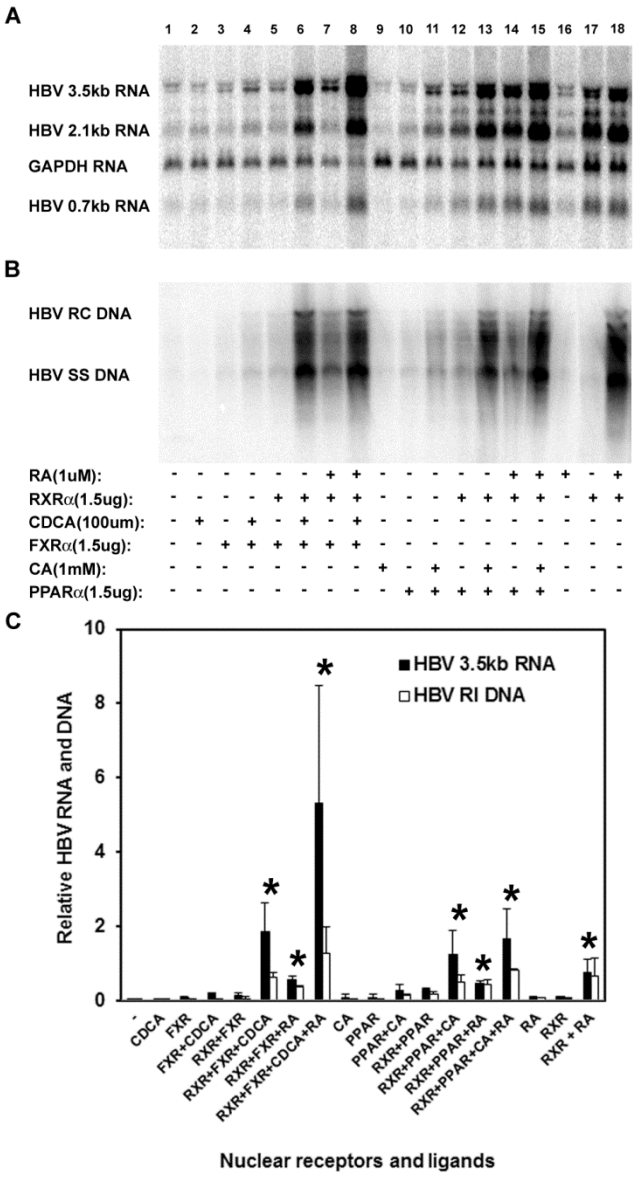

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622 Figure 4