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MULTIPLE NUCLEAR RECEPTORS MAY REGULATE HEPATITIS B VIRUS BIOSYNTHESIS DURING DEVELOPMENT

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Abstract

Hepatitis B virus (HBV) replicates by the reverse transcription of the viral 3.5kb pregenomic RNA. Therefore the level of expression of this transcript in the liver is a primary determinant of HBV biosynthesis. *In vivo* neonatal transcription of the HBV 3.5kb pregenomic RNA is developmentally regulated by hepatocyte nuclear factor 4 (HNF4). In addition, viral biosynthesis in non-hepatoma cells can be supported directly by this nuclear receptor. However HBV transcription and replication can be supported by additional nuclear receptors including the retinoid X receptor /peroxisome proliferator-activated receptor (RXR /PPAR), retinoid X receptor /farnesoid X receptor (RXR /FXR), liver receptor homolog 1 (LRH1) and estrogen-related receptors (ERR) in non-hepatoma cells. Therefore during neonatal liver development, HNF4 may progressively activate viral transcription and replication by binding directly to the proximal HNF4 recognition sequence within the nucleocapsid promoter. Alternatively, HNF4 may support viral biosynthesis *in vivo* indirectly by activating a network of liver-enriched nuclear receptors that, in combination, direct HBV 3.5kb pregenomic RNA transcription and replication.

Keywords

hepatitis B virus; nuclear receptors; transcription; replication; viral biosynthesis

INTRODUCTION

Hepatitis B virus (HBV) is a major human pathogen that chronically infects approximately 400 million individuals worldwide (Dienstag, 2008). Chronic HBV infections can lead to cirrhosis of the liver and hepatocellular carcinoma which are responsible for about one million deaths annually (Dienstag, 2008). HBV tropism is restricted to the liver of its hosts, man and chimpanzees, presumably because the viral receptor is expressed only on hepatocytes of these species (Raney and McLachlan, 1991). However studies of factors that control the transcription of the HBV genome have indicated that only liver-enriched nuclear receptors are capable of supporting HBV 3.5kb pregenomic RNA synthesis (Tang and McLachlan, 2001). Consequently expression of the viral genome is essentially limited to cells where these transcription factors are abundant, primarily the hepatocytes of the adult liver, adding another level of restriction to the tropism of this virus (Guidotti et al., 1995).

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In rodents, the abundance of the liver-enriched nuclear receptors that control HBV transcription is developmentally regulated. The levels of hepatocyte nuclear factor 4 (HNF4), retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR) and liver receptor homolog 1 (LRH1) in the liver increase as neonates develop into adults (Kyrnizi et al., 2006; Balasubramanian et al., 2005). Concomitantly, the level of HBV transcripts and replication intermediates are very low at birth in HBV transgenic mice and progressively increase throughout subsequent liver development (Guidotti et al., 1995; Li et al., 2009). As the HBV 3.5kb pregenomic RNA encodes the viral nucleocapsid and reverse transcriptase/DNA polymerase, the level of this transcript determines the abundance of the viral replication intermediates as it is also the substrate which is reverse transcribed into viral DNA (Will et al., 1987). The correlation of viral biosynthesis with nuclear receptor levels in the developing liver and the previous observation that HNF4 and RXR/PPAR can support HBV 3.5kb pregenomic RNA synthesis and replication in non-hepatoma cells raised the central issue of their relative importance for virion production in the liver *in vivo* (Tang and McLachlan, 2001; Balasubramanian et al., 2005; Kyrnizi et al., 2006; Li et al., 2009). To address this question, HBV transgenic mice lacking specific nuclear receptors have been characterized (Guidotti et al., 1999; Li et al., 2009). These studies indicate that PPAR does not affect viral biosynthesis under normal physiological conditions whereas HNF4 is essential for HBV transcription and replication throughout early neonatal development (Guidotti et al., 1999; Li et al., 2009). However the question of the precise mechanism of action of HNF4 throughout development remains to be established.

In this study, we investigate the role of several additional nuclear receptors in the control of HBV transcription and replication in non-hepatoma cells. Additional liver-enriched nuclear receptors have been shown to bind to and modulate nucleocapsid promoter activity (Li et al., 1998; Ishida et al., 2000; Ramiere et al., 2008). Therefore the effect of RXR/FXR and LRH1 plus the related nuclear receptor, estrogen-related receptors (ERR), on HBV 3.5kb pregenomic RNA synthesis and replication in non-hepatoma cells was examined and they were shown individually to have the capacity to support HBV biosynthesis. These observations, in combination with the expression pattern of these nuclear receptors in the developing liver, suggest that HBV has adapted to its host such that viral transcripts, replication intermediates and proteins are synthesized at progressively increasing levels after birth. This pattern of synthesis might induce immunological tolerance, supporting persistent infection, as observed in the majority of human neonatal infections (Dienstag, 2008).

2. Materials and methods

2.1. Plasmid constructions

The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (Sambrook et al., 1989). HBV DNA sequences in these constructions were derived from the plasmid pCP10, which contains two copies of the HBV genome (subtype *ayw*) cloned into the *EcoRI* site of pBR322 (Dubois et al., 1980). 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 (Fig. 1A). This plasmid was constructed by cloning the *NsiI/BglIII* HBV DNA fragment (nucleotide coordinates 1072 to 1990) into pUC13, generating pHBV(1072–1990). Subsequently, a complete copy of the 3.2kbp viral genome linearized at the *NcoI* site (nucleotide coordinates 1375 to 3182 plus 1 to 1374) was cloned into the unique *NcoI* site (HBV nucleotide coordinate 1374) of pHBV(1072–1990), generating the HBV DNA (4.1kbp) construct.

The pCMVHNF4, pRS-hRXR, pCMVPPAR-G, pCMV-rFXR, pCMX-mLRH1, pSG5-mERR, pcDNA3–2xFLAG-mERR and pSV5-mERR vectors express HNF4, RXR,

PPAR-G, FXR, LRH1, ERR, ERR and ERR polypeptides from the rat HNF4, human RXR, mouse PPAR-G, rat FXR, mouse LRH1, mouse ERR, mouse ERR and mouse ERR cDNAs, respectively, using the CMV immediate-early promoter (pCMV, pCMX and pcDNA3), the Rous sarcoma virus LTR (pRS) or the simian virus 40 early promoter (pSG5 and pSV) (Mangelsdorf et al., 1990; Muerhoff et al., 1992; Chen et al., 1994a; Bonnelye et al., 1997; Knutti et al., 2000; Lu et al., 2000). The PPAR-G polypeptide contains a mutation in the PPAR cDNA changing Glu²⁸² 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 (Muerhoff et al., 1992).

2.2 Cells and transfections

The human embryonic kidney 293T cell line was grown in RPMI-1640 medium and 10% fetal bovine serum at 37°C in 5% CO₂/air. Transfections for viral RNA and DNA analysis were performed as previously described (McLachlan et al., 1987) using 10 cm plates, containing approximately 1×10^6 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 µg of the nuclear receptor expression vectors, pCMVHNF4, pRS-hRXR, pCMVPPAR-G, pCMV-rFXR, pCMX-mLRH1, pSG5-ERR, pcDNA3-2xFLAG-mERR and pSV5-mERR vectors (Mangelsdorf et al., 1990; Muerhoff et al., 1992; Chen et al., 1994a; Bonnelye et al., 1997; Knutti et al., 2000; Lu et al., 2000). Controls were derived from cells transfected with HBV DNA and the expression vectors lacking a nuclear receptor cDNA insert (Raney et al., 1997). All-*trans* retinoic acid, clofibric acid and chenodeoxycholic acid at 1µM, 1mM and 100µM, respectively, were used to activate the nuclear receptors, RXR, PPAR and FXR (Tang and McLachlan, 2001).

2.3 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 (Summers et al., 1991) with minor modifications. For RNA isolation (Chomczynski and Sacchi, 1987) the cells were lysed in 1.8 ml of 25 mM sodium citrate, pH 7.0, 4 M guanidinium isothiocyanate, 0.5% (v/v) sarcosyl, 0.1 M 2-mercaptoethanol. After addition of 0.18 ml of 2M sodium acetate, pH 4.0, the lysate was extracted with 1.8 ml of water-saturated phenol plus 0.36 ml of chloroform-isoamyl alcohol (49:1). After centrifugation for 30 min. at 3,000 rpm in a Sorval RT6000, the aqueous layer was precipitated with 1.8 ml of isopropanol. The precipitate was resuspended in 0.3 ml of 25 mM sodium citrate, pH 7.0, 4 M guanidinium isothiocyanate, 0.5% (v/v) sarcosyl, 0.1 M 2-mercaptoethanol and precipitated with 0.6 ml of ethanol. After centrifugation for 20 min. at 14,000 rpm in an Eppendorf 5417C microcentrifuge, the precipitate was resuspended in 0.3 ml of 10 mM Tris hydrochloride, pH 8.0, 5 mM EDTA, 0.1% (w/v) sodium lauryl sulfate and precipitated with 45 µl of 2 M sodium acetate plus 0.7 ml of ethanol.

For the isolation of viral DNA replication intermediates, the cells were lysed in 0.4 ml of 100 mM Tris hydrochloride, pH 8.0, 0.2% (v/v) NP40. The lysate was centrifuged for 1 min. at 14,000 rpm in an Eppendorf 5417C microcentrifuge to pellet the nuclei. The supernatant was adjusted to 6.75 mM magnesium acetate plus 200 µg/ml DNase I and incubated for 1 hr at 37°C to remove the transfected plasmid DNA. The supernatant was readjusted to 100 mM NaCl, 10 mM EDTA, 0.8% (w/v) sodium lauryl sulfate, 1.6 mg/ml pronase and incubated for an additional 1 hr at 37°C. The supernatant was extracted twice with phenol, precipitated with two volumes of ethanol and resuspended in 100 µl of 10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA. 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 (Sambrook et al., 1989). Filter hybridization analyses were quantified by phosphorimaging using a Packard Cyclone Storage Phosphor System.

RESULTS

3.1. Multiple nuclear receptors can support HBV biosynthesis in nonhepatoma cells

A HBV DNA (4.1kbp) construct that can encode the four HBV transcripts and supports viral replication in hepatoma cells and the hepatocytes of transgenic mice was examined for its ability to support viral transcription and replication in the human embryonic kidney 293T cell line (Fig. 1) (Guidotti et al., 1995; Tang and McLachlan, 2001; Oropeza et al., 2008). In the absence of nuclear receptor expression vectors, the HBV 3.5kb pregenomic RNA is not expressed and viral replication is not apparent in transient transfection analysis (Fig. 1B and C, lane 1). HBV RNAs larger than 3.5kb of unknown origin are apparent and may represent greater-than-genome length transcripts originating from the X-gene promoter (Fig. 1B) (Doitsh and Shaul, 2003). The HBV 2.1kb RNA is expressed in the absence of nuclear receptors presumably because primary control of the major surface antigen promoter activity in transient transfection analysis is mediated by ubiquitous transcription factors (Raney et al., 1992; Raney and McLachlan, 1997; Tang and McLachlan, 2001). In contrast, HNF4, RXR/PPAR, RXR/FXR, LRH1, ERR and ERR, stimulate transcription of the HBV 3.5kb RNA and this is associated with the synthesis of encapsidated viral replication intermediates (Fig. 1B and C). ERR failed to stimulate transcription of the HBV 3.5kb RNA and did not support viral replication under these conditions in the human embryonic kidney 293T cell line (Fig. 1B and C, lane 6). These observations indicate that a number of different nuclear receptors can control the transcription of pregenomic RNA and therefore the ability of HBV to replicate in specific cell types.

To investigate further the mechanism of modulation of viral replication by the different nuclear receptors, a HBV DNA (4.1kbp) construct (Fig. 1A) with a 4-nucleotide mutation in the nucleocapsid promoter proximal HNF4 binding site (HNF4mut) was examined for its ability to support viral transcription and replication in the human embryonic kidney 293T cell line (Figs. 2 and 3A) (Tang and McLachlan, 2001). The mutation blocks nuclear receptor binding to the nucleocapsid promoter proximal HNF4 binding site and inhibits HNF4- and RXR/PPAR-mediated HBV 3.5kb pregenomic RNA synthesis and viral replication (Fig. 2, lanes 3–6) (Tang and McLachlan, 2001). The nuclear hormone receptor-dependent level of HBV 3.5kb RNA expressed from the HNF4mut construct was two- to four-fold lower than that observed from the wild type construct in the presence of expressed HNF4, RXR/PPAR and RXR/FXR (Fig. 2A, lanes 3–8) but changed only to a very limited extent with the expression of LRH1, ERR and ERR (Fig. 2A, lanes 9–10 and 13–16). As observed in mouse fibroblasts, HNF4- and RXR/PPAR-dependent viral replication derived from the HNF4 mutant construct was greatly reduced compared to the wild type construct indicating the importance of the proximal HNF4 binding site in the nucleocapsid promoter for the control of viral replication by these two nuclear receptors (Fig. 2B, lanes 3–6) (Tang and McLachlan, 2001). RXR/FXR-dependent viral replication derived from the HNF4 mutant construct was reduced approximately six-fold compared to the wild type construct indicating the importance of the proximal HNF4 binding site in the nucleocapsid promoter for the control of viral replication by this nuclear receptor although to a somewhat lesser extent than observed with HNF4 and RXR/PPAR (Fig. 2B, lanes 3–8). In contrast, LRH1-, ERR- and ERR-dependent viral replication derived from the HNF4 mutant construct was reduced only about two-fold compared to the wild type construct indicating the proximal HNF4 binding site in the nucleocapsid promoter has only a limited role in controlling the level of viral replication by these transcription factors (Fig. 2B, lanes 9–10 and 13–16). These findings indicate that the nucleocapsid promoter proximal

HNF4 binding site is essential for high levels of HBV 3.5kb pregenomic RNA synthesis by the dimeric nuclear receptors, HNF4 , RXR /PPAR and RXR /FXR , but is considerably less important for viral transcription and replication mediated by the monomeric nuclear receptors, LRH1, ERR and ERR . These findings imply that the activities of the various nuclear receptors differ, in part, due to their distinct abilities to promote transcription of the HBV 3.5kb pregenomic RNA from viral regulatory elements other than the nucleocapsid promoter proximal HNF4 binding site. Consequently, the level of viral replication is dependent on both the integrity of the nucleocapsid promoter and the nuclear receptors controlling viral gene expression. As noted previously, the effect of mutation of the nucleocapsid promoter proximal HNF4 binding site on replication is greater than its effect on transcription because it preferentially reduces the level of the HBV 3.5kb pregenomic RNA compared with the HBV 3.5kb precore RNA (Tang and McLachlan, 2001).

DISCUSSION

4.1 Nuclear receptor binding sites in the HBV nucleocapsid promoter

The transcription factors that bind to the nucleocapsid promoter and regulate the level of expression of the HBV 3.5kb RNA have been extensively characterized (Fig. 3) (Tang et al., 2001). A variety of ubiquitous and liver-enriched transcription factors have been identified that contribute to nucleocapsid promoter activity in reporter gene assays but the nuclear receptors, HNF4 and RXR /PPAR , are the only transcription factors that have been reported previously to support efficient HBV 3.5kb pregenomic RNA synthesis and viral replication in non-hepatoma cells (Tang and McLachlan, 2001). However, additional nuclear receptors have been identified that bind to and regulate nucleocapsid promoter activity in hepatoma cells. Notably, FXR and LRH1 were found to modulate HBV nucleocapsid promoter activity (Li et al., 1998; Ishida et al., 2000; Ramiere et al., 2008). As the recognition sequence of ERR is very similar to LRH1 (Tsukiyama and Niwa, 1992; Johnston et al., 1997), we surmised that this nuclear receptor might also bind to the nucleocapsid promoter. Therefore, the ability of RXR /FXR , LRH1 and ERR to support HBV 3.5kb pregenomic RNA synthesis and viral replication in non-hepatoma cells was determined (Fig. 1). From this analysis, it was apparent that RXR /FXR , LRH1 and ERR, in addition to HNF4 and RXR /PPAR , can direct the efficient expression of the HBV 3.5kb pregenomic RNA and support viral replication in non-hepatoma cells. These findings add an additional level of complexity to the transcriptional regulation of HBV biosynthesis.

4.2 Location of nuclear receptor binding sites within the HBV nucleocapsid promoter

The observation that multiple nuclear receptors can support transcription from the nucleocapsid promoter raised the issue of the importance of the proximal HNF4 recognition site for their activities. Previously it had been shown that the majority of the HNF4 - and RXR /PPAR -dependent replication in mouse NIH 3T3 fibroblasts was mediated through the proximal HNF4 recognition site within the nucleocapsid promoter (Tang and McLachlan, 2001). Similar findings were observed using the human embryonic kidney 293T cell line and the dimeric nuclear receptors, HNF4 , RXR /PPAR and RXR /FXR (Fig. 2). In contrast, only about a two-fold reduction in viral replication was observed in the human embryonic kidney 293T cell line when the monomeric nuclear receptors, LRH1, ERR and ERR directed the expression of the HBV 3.5kb pregenomic RNA (Fig. 2). These observations indicate that the monomeric nuclear receptors must interact with the proximal HNF4 recognition site within the nucleocapsid promoter to mediate part of their effect on viral biosynthesis. In addition, there must be other nuclear receptor binding sites within the viral genome for these transcription factors that contribute approximately equally to mediating these effects on HBV transcription and replication (Fig. 3).

Two RXR /FXR binding sites have been identified within the nucleocapsid promoter (Fig. 3A) (Ramieri et al., 2008). Therefore it appears that binding to the proximal RXR /FXR recognition site, which overlaps with the proximal HNF4 recognition site, within the nucleocapsid promoter contributes the majority of the transcriptional activity whereas binding to the distal site contributes the remaining RXR /FXR-mediated promoter activity. These observations indicate that the dimeric nuclear receptors, HNF4, RXR /PPAR and RXR /FXR, efficiently support viral biosynthesis when they are bound to the proximal nuclear receptor binding sites within the nucleocapsid promoter and only poorly support HBV transcription and replication when bound at distal recognition sites (Figs. 2 and 3A).

Two LRH1 binding sites have been previously identified in the distal nucleocapsid promoter sequences (Li et al., 1998; Ishida et al., 2000). As mutation of the proximal HNF4 recognition site within the nucleocapsid promoter reduces LRH1-mediated HBV biosynthesis approximately in half, it appears that an additional recognition sequence for this nuclear receptor must exist within the proximal HNF4 recognition site. This recognition sequence probably includes the second direct repeat of the HNF4 binding site as the TAAAGGTCT sequence most closely matches the LRH1 consensus sequence, YCAAGGYCR (Tsukiyama and Niwa, 1992). Therefore the remaining promoter activity must be governed by the two distal LRH1 binding sites within the nucleocapsid promoter (Fig. 3B) (Li et al., 1998; Ishida et al., 2000).

As the consensus sequence for LRH1 is very similar to the consensus binding site sequence for ERR, TCAAGGTCA (Johnston et al., 1997), it was of interest to determine if ERR might also support HBV biosynthesis in non-hepatoma cells. In the human embryonic kidney 293T cell line, ERR and ERR support viral biosynthesis whereas ERR failed to mediate detectable levels of HBV replication (Figs. 1C and 2B). However, complementation with coactivators demonstrated that all the ERR isoforms have the potential to support viral biosynthesis (Rushing and McLachlan, unpublished results). Mutation of the proximal HNF4 recognition site within the nucleocapsid promoter decreases both ERR- and ERR-dependent HBV replication approximately two-fold indicating that additional ERR recognition sites must exist within the distal promoter sequences. The distal ERR binding sites likely correspond to the LRH1 binding sites (Fig. 3B). Indeed the locations of the ERR binding sites predicted using Consite (<http://asp.iu.uib.no:8090/cgi-bin/CONSITE/consite/>) software, in combination with the appropriate transcription factor weight matrix data, identified the distal LRH1 binding site at approximately nucleotide coordinate 1650 and the second direct repeat of the proximal HNF4 binding site at approximately nucleotide coordinate 1765 as the most likely ERR recognition sequences within the nucleocapsid promoter (Fig. 3A). Therefore it appears that approximately half the transcriptional activity mediated by ERR is due to binding to distal recognition sites within the nucleocapsid promoter and the remainder is mediated through the proximal binding site which can functionally interact with several nuclear receptor including ERR, LRH1, RXR /FXR, RXR /PPAR and HNF4 (Fig. 3A).

4.3 In vivo role of nuclear receptors in the biosynthesis of HBV during development

Analysis of HBV biosynthesis in non-hepatoma cells has demonstrated that nuclear receptors are the only transcription factors capable of supporting HBV 3.5kb pregenomic RNA synthesis and viral replication (Fig. 1) (Tang and McLachlan, 2001). In fact the nucleocapsid promoter contains nine direct repeat sequence elements related to the hexameric nuclear receptor recognition sequence, AGGTCA, within 250 nucleotides of the initiation sites of the HBV 3.5kb RNAs (Fig. 3A) (Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995). This observation suggests that the *in vivo* role of nuclear receptors in HBV biosynthesis is probably complex. This is supported by the finding that multiple

nuclear receptors, interacting with several different recognition elements within the nucleocapsid promoter, can support viral replication in non-hepatoma cells (Figs. 1–Fig. 3). As only man and chimpanzee are highly susceptible to HBV infection, the only small animal model of chronic HBV infection readily amenable to studying the *in vivo* role of nuclear receptors in the viral life cycle is the HBV transgenic mouse (Guidotti et al., 1995). Based on our cell culture studies and using the HBV transgenic mouse model of chronic HBV infection, the role of PPAR α in HBV biosynthesis was investigated *in vivo* (Guidotti et al., 1999). This analysis demonstrated that PPAR α did not play a role in determining the level of viral biosynthesis in the liver under normal physiological conditions (Guidotti et al., 1999). However, treatment of HBV transgenic mice with peroxisome proliferators enhanced viral transcription and replication in a PPAR α -dependent manner indicating that this nuclear receptor can activate viral biosynthesis under certain circumstances (Guidotti et al., 1999).

The second nuclear receptor examined in the HBV transgenic mouse model of chronic infection was HNF4 α (Li et al., 2009). HNF4 α is a transcription factor that is essential for the viability of the mouse (Chen et al., 1994b; Hayhurst et al., 2001). However utilizing the conditional tissue-specific deletion of HNF4 α in the neonatal liver, it was possible to determine the role of this nuclear receptor in HBV biosynthesis during early postnatal development (Li et al., 2009). Loss of HNF4 α expression resulted in the absence of HBV transcription and replication in the livers of the HBV transgenic mice demonstrating that HNF4 α is essential for viral biosynthesis *in vivo* (Li et al., 2009). This finding supports the analysis in non-hepatoma cells where HNF4 α binding to the proximal recognition sequence within the nucleocapsid promoter is essential for HBV 3.5kb pregenomic RNA synthesis and viral replication (Figs. 1 and 2) (Tang and McLachlan, 2001).

Although HNF4 α is essential for viral biosynthesis in early neonatal development in the HBV transgenic mouse, it is still not established if the effects of HNF4 α *in vivo* are direct or indirect. However, it is apparent that the effect of HNF4 α occurs at the level of viral transcription as the loss of this nuclear receptor is associated with a corresponding decrease in HBV transcription (Li et al., 2009). If the effect of HNF4 α is not directly on viral transcription, it most likely affects this process by altering the activities of additional nuclear receptors that can support HBV biosynthesis (Fig. 1) which, like HNF4 α , are involved in the complex transcription factor regulatory network required to produce and maintain the differentiated hepatic phenotype (Kymizi et al., 2006). As loss of PPAR α does not affect HBV biosynthesis under normal physiological conditions, it seems most likely that LRH1 and FXR represent the alternative liver-enriched nuclear receptors that might direct HBV transcription and replication. Indeed, HNF4 α is known to modulate the levels of expression of LRH1, PPAR α and FXR during early neonatal development (Fig. 3B) (Pineda Torra et al., 2002; Balasubramanian et al., 2005; Kymizi et al., 2006). Therefore, it is possible that HNF4 α controls HBV biosynthesis indirectly by increasing the expression of LRH1, PPAR α and FXR during early neonatal development and thereby indirectly activating HBV replication. To examine this possibility, HBV transgenic mice lacking LRH1 or FXR in the liver will have to be characterized for their ability to support viral transcription and replication throughout postnatal development.

The potential role of ERR in HBV biosynthesis is less apparent. ERR α is not expressed in the liver and therefore is unlikely to contribute to viral transcription (Bookout et al., 2006). Likewise, ERR β and ERR γ are expressed in the liver at modest levels but are also expressed in a variety of other tissues that have not been shown to support viral biosynthesis (Bookout et al., 2006). These observations suggest ERR may not contribute directly to HBV biosynthesis although it may enhance the activities of other nuclear receptors either directly by binding to the nucleocapsid promoter or possibly indirectly by modulating PPAR α , and possibly HNF4 α , activity within the liver (Fig. 3B).

4.4 Adaptation of HBV to host development as an effective strategy for viral persistence

The parallel increase in HNF4 expression and HBV biosynthesis levels observed during postnatal development suggest that neonatal HBV infection in man may be associated with a slow, developmentally regulated increase in viral transcription, replication and antigen expression. This may contribute to host immunological tolerance to viral antigens and subsequent life long chronic infection (Milich et al., 1990; Dienstag, 2008). In addition, the essential nature of the HNF4 transcription factor for host viability (Chen et al., 1994b; Hayhurst et al., 2001; Parviz et al., 2003) probably ensures that HBV transcription and replication cannot be resolved without killing infected hepatocytes further limiting the hosts' ability to resolve infection and increasing the probability of viral persistence. This adaptation may have a significant selective advantage and help to explain the success of this pathogen that currently chronically infects approximately 400 million individuals worldwide (Dienstag, 2008).

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REFERENCES

- Bailly A, Torres-Padilla ME, Tinel AP, Weiss MC. An enhancer element 6 kb upstream of the mouse HNF4 1 promoter is activated by glucocorticoids and liver-enriched transcription factors. *Nucleic Acids Research*. 2001; 29:3495–3505. [PubMed: 11522818]
- Balasubramaniyan N, Shahid M, Suchy FJ, Ananthanarayanan M. Multiple mechanisms of ontogenic regulation of nuclear receptors during rat liver development. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2005; 288:G251–G260. [PubMed: 15388488]
- Bonnelye E, Vanacker JM, Dittmar T, Begue A, Desbiens X, Denhardt DT, Aubin JE, Laudet V, Fournier B. The ERR-1 orphan receptor is a transcriptional activator expressed during bone development. *Molecular Endocrinology*. 1997; 11:905–916. [PubMed: 9178750]
- Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell*. 2006; 126:789–799. [PubMed: 16923397]
- Chen D, Lepar G, Kemper B. A transcriptional regulatory element common to a large family of hepatic cytochrome P450 genes is a functional binding site of the orphan receptor HNF-4. *Journal of Biological Chemistry*. 1994a; 269:5420–5427. [PubMed: 8106524]
- Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE Jr. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes and Development*. 1994b; 8:2466–2477. [PubMed: 7958910]
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*. 1987; 162:156–159. [PubMed: 2440339]
- Dienstag JL. Hepatitis B virus infection. *N. Engl. J. Med*. 2008; 359:1486–1500. [PubMed: 18832247]
- Doitsh G, Shaul Y. A long HBV transcript encoding pX is inefficiently exported from the nucleus. *Virology*. 2003; 309:339–349. [PubMed: 12758180]
- Dubois MF, Pourcel C, Rousset S, Chany C, Tiollais P. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. *Proceedings of the National Academy of Sciences of the United States of America*. 1980; 77:4549–4553. [PubMed: 6933503]

- Guidotti LG, Eggers CM, Raney AK, Chi SY, Peters JM, Gonzalez FJ, McLachlan A. *In vivo* regulation of hepatitis B virus replication by peroxisome proliferators. *Journal of Virology*. 1999; 73:10377–10386. [PubMed: 10559356]
- Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. *Journal of Virology*. 1995; 69:6158–6169. [PubMed: 7666518]
- Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor 4 (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Molecular and Cellular Biology*. 2001; 21:1393–1403. [PubMed: 11158324]
- Huss JM, Torra IP, Staels B, Giguere V, Kelly DP. Estrogen-related receptor directs peroxisome proliferator-activated receptor signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. *Molecular and Cellular Biology*. 2004; 24:9079–9091. [PubMed: 15456881]
- Ishida H, Ueda K, Ohkawa K, Kanazawa Y, Hosui A, Nakanishi F, Mita E, Kasahara A, Sasaki Y, Hori M, Hayashi N. Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. *Journal of Virology*. 2000; 74:1241–1251. [PubMed: 10627534]
- Johnston SD, Liu X, Zuo F, Eisenbraun TL, Wiley SR, Kraus RJ, Mertz JE. Estrogen-related receptor 1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. *Molecular Endocrinology*. 1997; 11:342–352. [PubMed: 9058380]
- Knutti D, Kaul A, Kralli A. A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. *Molecular and Cellular Biology*. 2000; 20:2411–2422. [PubMed: 10713165]
- Kymrzi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes and Development*. 2006; 20:2293–2305. [PubMed: 16912278]
- Li L, Oropeza CE, Sainz B Jr, Uprichard SL, Gonzalez FJ, McLachlan A. Developmental regulation of hepatitis B virus biosynthesis by hepatocyte nuclear factor 4. *Public Library of Science ONE*. 2009 in press.
- Li M, Xie YH, Kong YY, Wu X, Zhu L, Wang Y. Cloning and characterization of a novel human hepatocyte transcription factor, hB1F, which binds and activates enhancer II of hepatitis B virus. *Journal of Biological Chemistry*. 1998; 273:29022–29031. [PubMed: 9786908]
- Liu D, Zhang Z, Teng CT. Estrogen-related receptor- and peroxisome proliferator-activated receptor-coactivator-1 regulate estrogen-related receptor- gene expression via a conserved multi-hormone response element. *J. Molecular Endocrinology*. 2005; 34:473–487.
- Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Molecular Cell*. 2000; 6:507–515. [PubMed: 11030331]
- Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. *Cell*. 1995; 83:841–850. [PubMed: 8521508]
- Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature*. 1990; 345:224–229. [PubMed: 2159111]
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: The second decade. *Cell*. 1995; 83:835–839. [PubMed: 8521507]
- McLachlan A, Milich DR, Raney AK, Riggs MG, Hughes JL, Sorge J, Chisari FV. Expression of hepatitis B virus surface and core antigens: Influences of pre-s and precore sequences. *Journal of Virology*. 1987; 61:683–692. [PubMed: 3543403]
- Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance *in utero*? *Proceedings of the National Academy of Sciences of the United States of America*. 1990; 87:6599–6603. [PubMed: 2395863]
- Muerhoff AS, Griffin KJ, Johnson EF. The peroxisome proliferator-activated receptor mediates the induction of *CYP4A6*, a cytochrome P450 fatty acid omega-hydroxylase, by clofibrilic acid. *Journal of Biological Chemistry*. 1992; 267:19051–19053. [PubMed: 1326542]

- Oropeza CE, Li L, McLachlan A. Differential inhibition of nuclear hormone receptor dependent hepatitis B virus replication by small heterodimer partner. *Journal of Virology*. 2008; 82:3814–3821. [PubMed: 18234786]
- Paré JF, Roy S, Galarneau L, Bélanger L. The mouse fetoprotein transcription factor (FTF) gene promoter is regulated by three GATA elements with tandem E box and Nkx motifs, and FTF in turn activates the *Hnf3*, *Hnf4*, and *Hnf1* gene promoters. *Journal of Biological Chemistry*. 2001; 276:13136–13144. [PubMed: 11145965]
- Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA. Hepatocyte nuclear factor 4 controls the development of a hepatic epithelium and liver morphogenesis. *Nature Genetics*. 2003; 34:292–296. [PubMed: 12808453]
- Pineda Torra I, Claudel T, Duval C, Kosykh V, Fruchart JC, Staels B. Bile acids induce the expression of the human peroxisome proliferator-activated receptor gene via activation of the farnesoid X receptor. *Molecular Endocrinology*. 2003; 17:259–272. [PubMed: 12554753]
- Pineda Torra I, Jamshidi Y, Flavell DM, Fruchart JC, Staels B. Characterization of the human PPAR promoter: Identification of a functional nuclear receptor response element. *Molecular Endocrinology*. 2002; 16:1013–1028. [PubMed: 11981036]
- Ramire C, Scholtes C, Diaz O, Icard V, Perrin-Cocon L, Trabaud MA, Lotteau V, Andre P. Transactivation of the hepatitis B virus core promoter by the nuclear receptor FXR. *Journal of Virology*. 2008; 82:10832–10840. [PubMed: 18768987]
- Raney AK, Johnson JL, Palmer CNA, McLachlan A. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. *Journal of Virology*. 1997; 71:1058–1071. [PubMed: 8995626]
- Raney AK, Le HB, McLachlan A. Regulation of transcription from the hepatitis B virus major surface antigen promoter by the Sp1 transcription factor. *Journal of Virology*. 1992; 66:6912–6921. [PubMed: 1331502]
- Raney, AK.; McLachlan, A. The biology of hepatitis B virus. In *Molecular biology of the hepatitis. virus, B.*; McLachlan, A., editors. Boca Raton, Florida: CRC Press; 1991. p. 1-37.
- Raney AK, McLachlan A. Characterization of the hepatitis B virus major surface antigen promoter hepatocyte nuclear factor 3 binding site. *Journal of General Virology*. 1997; 78:3029–3038. [PubMed: 9367390]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press; 1989.
- Summers J, Smith PM, Huang M, Yu M. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. *Journal of Virology*. 1991; 65:1310–1317. [PubMed: 1995945]
- Tang H, Banks KE, Anderson AL, McLachlan A. Hepatitis B virus transcription and replication. *Drug News and Perspectives*. 2001; 14:325–334. [PubMed: 12813595]
- Tang H, McLachlan A. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:1841–1846. [PubMed: 11172038]
- Tsukiyama T, Niwa O. Isolation of high affinity cellular targets of the embryonal LTR binding protein, an undifferentiated embryonal carcinoma cell-specific repressor of Moloney leukemia virus. *Nucleic Acids Research*. 1992; 20:1477–1482. [PubMed: 1579438]
- Will H, Reiser W, Weimer T, Pfaff E, Buscher M, Sprengle R, Cattaneo R, Schaller H. Replication strategy of human hepatitis B virus. *Journal of Virology*. 1987; 61:904–911. [PubMed: 3806799]
- Zhang YQ, Castellani LW, Sinal CJ, Gonzalez FJ, Edwards PA. Peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1) regulates triglyceride metabolism by activation of the nuclear receptor FXR. *Genes and Development*. 2004; 18:157–169. [PubMed: 14729567]
- Zhang Z, Teng CT. Interplay between estrogen-related receptor alpha (ERR) and gamma (ERR) on the regulation of ERR gene expression. *Molecular and Cellular Endocrinology*. 2007; 264:128–141. [PubMed: 17157980]

Figure 1.

Multiple nuclear receptors activate HBV transcription and replication in the human embryonic kidney 293T cell line. (A) Structure of the HBV DNA (4.1kbp) construct used in transient transfection analysis. The 4.1-kbp greater-than-genome length HBV DNA sequence in this construct spans coordinates 1072-3182/1-1990 of the HBV genome (subtype *ayw*). The locations of the HBV 3.5-kb, 2.4-kb, 2.1-kb and 0.7-kb transcripts are indicated. EnhI/Xp, enhancer I/X-gene promoter region; Cp, nucleocapsid or core promoter; pA, polyadenylation site; PS1p, presurface antigen promoter; Sp, major surface antigen promoter; X, X-gene; S, surface antigen gene; C, core gene; P, polymerase gene; ORF, open reading frame. (B) 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. (C) DNA (Southern) filter hybridization analysis of HBV replication intermediates. HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single stranded DNA. All-*trans* retinoic acid, clofibrate and chenodeoxycholic acid at 1 μ M, 1mM, and 100 μ M, respectively, were used to activate the nuclear receptors, RXR , PPAR and FXR . Cells were transiently transfected with the HBV DNA (4.1kbp) construct and the indicated nuclear receptor expression vectors. Lane 1, Control; lane 2, HNF4 ; lane 3, RXR plus PPAR ; lane 4, RXR plus FXR ; lane 5, LRH1; lane 6, ERR ; lane 6, ERR ; lane 8, ERR .

Figure 2.

The nucleocapsid promoter proximal HNF4 binding site is an important determinant of nuclear receptor mediated HBV replication in the human embryonic kidney 293T cell line. Cells were transiently transfected with wild type (wt) or mutant (mt) HBV DNA (4.1kbp) constructs and nuclear receptors. Lanes 1–2, Control; lanes 3–4, HNF4 ; lane 5–6, RXR plus PPAR ; lanes 7–8, RXR plus FXR ; lanes 9–10, LRH1; lanes 11–12, ERR ; lanes 13–14, ERR ; lanes 15–16, ERR . The HBV HNF4mut DNA (4.1kbp) construct contains the 4-nucleotide mutation (Fig. 3A) in the proximal HNF4 binding site of the nucleocapsid promoter that inhibits the binding of nuclear receptors to this recognition sequence (Tang and McLachlan, 2001). The nucleotide substitutions do not alter the X-gene polypeptide sequence. Both nucleocapsid promoter proximal regions in this terminally redundant HBV construct (Fig. 1A) were mutated for this analysis. (B) RNA (Northern) filter hybridization analysis of HBV transcripts. (C) DNA (Southern) filter hybridization analysis of HBV replication intermediates.

Figure 3.

(A) Sequence of the HBV nucleocapsid promoter region. The 4-nucleotide HNF4 site mutation indicated above the wild type sequence in the green boxes inhibits the binding of nuclear receptors to the proximal HNF4 binding site (Tang and McLachlan, 2001). The nucleotide substitutions do not alter the X-gene polypeptide sequence. The RFX1, Sp1, C/EBP, HNF3 and TBP recognition sequences are underlined (Tang et al., 2001). The nine direct repeat sequence elements related to the hexameric nuclear receptor recognition sequence, AGGTCA, within the nucleocapsid promoter are highlighted in yellow. The identified or presumptive recognition sequences for the nuclear receptors, HNF4 (Raney et al., 1997), RXR /PPAR (Raney et al., 1997), RXR /FXR (Ramieri et al., 2008), LRH1

(Li et al., 1998; Ishida et al., 2000) and ERR, are indicated in blue. The initiation sites for the HBV precore (PC) and pregenomic (or core (C)) 3.5kb RNAs are also indicated. (B) Gene regulatory network interactions among nuclear receptors within the liver that regulate HBV biosynthesis. HNF4, LRH1, PPAR and ERR can auto-regulate their own expression (Bailly et al., 2001; Paré et al., 2001; Pineda Torra et al., 2002; Liu et al., 2005; Zhang and Teng, 2007). HNF4 regulates the expression of LRH1, PPAR and FXR (Paré et al., 2001; Pineda Torra et al., 2002; Zhang et al., 2004). LRH1 can activate HNF4 transcription (Paré et al., 2001). PPAR reciprocally regulates both FXR and ERR expression (Pineda Torra et al., 2003; Huss et al., 2004; Zhang et al., 2004; Liu et al., 2005). ERR regulates ERR expression (Liu et al., 2005). Additionally interactions within this regulatory network controlling HBV transcription probably also exist. In particular, it is likely that LRH1 and ERR can reciprocally regulate each others expression given the close similarity in their recognition sequences. For similar reasons, ERR may contribute to HNF4 expression. However, early in neonatal development HNF4 is essential, either directly or indirectly, for HBV biosynthesis (Kymizi et al., 2006; Li et al., 2009).