Transcriptional Regulation of Hepatitis B Virus Biosynthesis by Bile Acids and FoxA Factors

BY
VANESSA REESE
BA, Brown University, 2006

THESIS

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Chicago, Illinois

Defense Committee:
Alan McLachlan, Chair and Advisor
Susan Uprichard
Nancy Freitag
Deepak Shukla
Karen Colley, Biochemistry and Molecular Genetics
To my husband,

Ben McFadden,

whose support makes everything possible

and who I cannot wait to spend the rest of my life with
I want to thank Alan and Claudia. I want to thank my committee and the MSTP Program. A special thanks to my wonderful family and all of my friends who helped me in this process.

VR
Chapter 1 is a literature review that places my dissertation question in the context of the larger field and highlights the significance of my research question. Chapter 2 describes the methods used during my thesis work. Chapter 3 represents work from published manuscripts: 1) Ondracek CR, Rushing CN, Reese VC, Oropeza CE, McLachlan A. Peroxisome proliferator-activated receptor gamma Coactivator 1alpha and small heterodimer partner differentially regulate nuclear receptor dependent hepatitis B virus biosynthesis. J Virol. 2009 Dec;83(23):12535-44; 2) Ondracek CR, Reese VC, Rushing CN, Oropeza CE, McLachlan A. Distinct regulation of hepatitis B virus biosynthesis by peroxisome proliferator-activated receptor gamma coactivator 1alpha and small heterodimer partner in human hepatoma cell lines. J Virol. 2009 Dec;83(23):12545-51; 3) Reese VC, Moore DD, McLachlan A. Limited effects of bile acids and small heterodimer partner on hepatitis B virus biosynthesis in vivo. J Virol. 2012 Mar;86(5):2760-8. In all of these I was a primary author and major driver of the research. My research mentor, Dr. Alan McLachlan contributed to the writing of these manuscripts. My work was critical to the conclusions of these manuscripts because they are based on the experiments I not only carried out, but also helped plan. Chapter 4 represents a series of my own unpublished experiments directed at answering the question of the role of FoxA in HBV biosynthesis. I anticipate that this line of research will be continued in the laboratory after I leave, and that this work will ultimately be published as part of a co-authored manuscript. Chapter 5 represents my synthesis of the research presented in this dissertation and my overarching conclusions. The future directions of this field and this research question are discussed.
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LIST OF ABBREVIATIONS

DNA          Deyoxyribonucleic Acid
RNA          Ribonucleic Acid
HBV          Hepatitis B Virus
HCC          Hepatocellular Carcinoma
CCC          Covalently Closed Circular
HNF4α        Hepatocyte Nuclear Factor 4α
RXRα         Retinoid X Receptor α
PPARα        Peroxisome Proliferators-Activated Receptor α
FXRα         Farnesoid X Receptor α
LXR          Liver X Receptor
LRH 1        Liver Receptor Homolog 1
ERR          Estrogen Related Receptor
CYP7A1       Cytochrome P450 7A1
BSEP         Bile Salt Export Pump
SHP          Small Heterodimer Partner
HNF3         Hepatocyte Nuclear Factor 3
ChIP         Chromatin Immunoprecipitation
GR           Glucocorticoid Receptor
Pck1         Phosphoenolpyruvate Carboxykinase 1
Igfbp1       Insulin-like Growth Factor-Binding Protein 1
CA           Cholic Acid
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
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<tr>
<td>αSMA</td>
<td>α Smooth Muscle Actin</td>
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<tr>
<td>AFP</td>
<td>Alpha Fetoprotein</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
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<tr>
<td>20AS</td>
<td>2’-5’-oligodenylate synthetase</td>
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<td>CK19</td>
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<td>TNFα</td>
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<td>Hepatitis B Virus E Antigen</td>
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<td>ALT</td>
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<td>HBcAg</td>
<td>Hepatitis B Virus Core Antigen</td>
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<tr>
<td>IFNα/β</td>
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<td>IFNγ</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>polyI:C</td>
<td>Polyninosinic:polycytidylic acid</td>
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<tr>
<td>CCl₄</td>
<td>Carbon Tetrachloride</td>
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SUMMARY

HBV is a major human pathogen that currently chronically infects approximately 400 million individuals worldwide and is responsible for about one million deaths annually. New modalities of therapy are urgently needed to address this major public health issue. The results presented show multiple nuclear receptors are capable of activating HBV biosynthesis in non-hepatoma cells, including RXRα/FXRα, and both SHP and PGC1α can modulate RXRα/FXRα-dependent HBV biosynthesis in non-hepatoma cells. However, bile acids have only a modest effect in vivo. Based on the cell culture analysis, it is possible that in vivo even the activation of FXR by bile acids is not sufficient to further enhance HBV transcription and replication, which is being directed by other constitutively active nuclear receptors. Of great interest, the results presented suggest that FoxA transcription factor deficiency in vivo can selectively prevent HBV biosynthesis without greatly altering hepatocyte physiology. However significant fibrosis, a common consequence of HBV infection, is apparent in the FoxA-deficient mice. It remains to be determined what the relative contributions from direct transcriptional regulation by FoxA and indirect effects mediated by nonparanchymal cells are to HBV biosynthesis in this system. Although findings presented in this study suggest FoxA deficiency itself can result in inhibition of HBV biosynthesis, it is quite possible both modes of regulation contribute to the observed effects on HBV biosynthesis.
1. INTRODUCTION

1.1 Hepatitis B Virus (HBV)

1.1.1 HBV Disease

Although the first HBV vaccine was made available in 1982, HBV continues to place a substantial disease burden on both the United States and global populations (17, 24). Worldwide approximately 2 billion people have been infected, of which approximately 400 million people are chronically infected, and in the United States alone, more than 1 million people are chronically infected. Each year about 1 million people die worldwide from the consequences of HBV infection, including cirrhosis, liver failure and hepatocellular carcinoma (HCC) (17, 24). These clinical consequences can be acute or chronic and range from the sub-clinical to fatal forms of the disease, including fulminant hepatitis where the patient dies shortly after infection. Therefore, there is a significant demand for novel treatment options for patients chronically infected with HBV. The estimated risk of HCC in individuals chronically infected with HBV is approximately 100 times greater than uninfected individuals. HBV replicates by reverse transcription of the viral pregenomic 3.5-kb RNA encoded by the HBV 3.2-kb genome (85, 97). Thus, understanding the mechanism regulating hepatitis B virus transcription and replication may reveal potential targets for antiviral therapy.

1.1.2 HBV Virology

HBV is an enveloped, partially double stranded DNA virus with four open reading frames (82). HBV transcription is regulated by four promoters and two
enhancer regions. The core promoter initiates the synthesis of the pregenomic and precore 3.5kb RNAs (103). Therefore, this promoter plays a crucial role in the HBV life cycle, because the pregenomic RNA encodes both the core protein and reverse transcriptase/DNA polymerase, and it is the substrate that is reverse transcribed into viral DNA (82). The precore RNA encodes the precore protein, also known as the HBe antigen.

The HBV life cycle is depicted in Figure 1. The replication strategy of HBV requires the 3.5-kb pregenomic RNA, which is transcribed from nuclear HBV covalently closed circular (CCC) DNA, serve an additional function as the replication template in the viral life cycle, besides encoding the nucleocapsid proteins and the HBV DNA polymerase (97). This RNA and the product of the HBV DNA polymerase open reading frame are encapsidated in the cytoplasm by the nucleocapsid proteins to form immature core particles (33). The reverse transcriptase and RNase H activities of the HBV DNA polymerase convert the pregenomic 3.5-kb RNA into the 3.2-kb partially double-stranded HBV genome found in the virus particle (33). The final step of the assembly of the virus particle involves the association of the mature core particle with an appropriately arranged assembly of envelope antigen molecules within the endoplasmic reticulum membrane. The surface antigen assembly subsequently buds into the lumen of the endoplasmic reticulum enveloping the mature core particle (73, 75). The complete virion is secreted from the cell through the endoplasmic reticulum and Golgi apparatus pathway.
Figure 1. **HBV life cycle.** The HBV replication cycle showing the intracellular pathway for the synthesis and secretion of HBV, HBsAg and HBeAg polypeptides.
1.1.3 **HBV Tropism**

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, although the entry mechanism and proteins involved have not been defined (25, 74). Additionally, HBV tissue tropism is restricted at the level of transcription (29). Studies of factors that control the transcription of the HBV genome have indicated that only liver-enriched nuclear receptors are capable of supporting HBV 3.5-kb pregenomic RNA synthesis in nonhepatoma cells (89). Consequently expression of the viral genome is essentially limited to cells where these transcription factors are abundant, primarily the hepatocytes of the adult liver (29). HBV is capable of replication in differentiated hepatoma cell lines, but replication cannot occur in nonhepatoma cell lines without appropriate complementation. Studies of HBV transcription have demonstrated that certain liver-enriched nuclear receptors are able to support HBV biosynthesis in nonhepatoma cells (34, 35, 77, 89).

1.1.4 **Nuclear Receptor Regulation of HBV**

Nuclear receptors are transcription factors and are responsible for a wide range of processes throughout the body (8, 59, 84). In the liver, some important nuclear receptors are hepatocyte nuclear factor 4α (HNF4α), retinoid X receptor α (RXRα), peroxisome proliferators-activated receptor α (PPARα), and farnesoid X receptor α (FXRα). HNF4α is a master regulator of liver gluconeogenesis and regulates the expression of other nuclear receptors (79). In metabolic tissues
RXRα serves as the heterodimeric partner for other nuclear receptors, including PPARα and FXRα (83). PPARα is activated by long chain fatty acids and plays a critical role in fat and carbohydrate metabolism (38). FXRα is activated by bile acids and is critical in regulating bile acid metabolism (23, 58). Multiple transcription factor binding sites have been identified on the HBV promoters and enhancers, and some nuclear receptors have been shown to be capable of mediating HBV biosynthesis and likely contribute to HBV liver tropism (3, 10, 52, 71, 77, 89, 90, 106). Non-hepatoma cells do not support HBV biosynthesis, however, exogenous expression of these nuclear receptors is sufficient for HBV transcription and replication. 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 (59, 61, 104). This observation suggests that the in vivo role of nuclear receptors in HBV biosynthesis is most likely complex (Fig 2).
Figure 2. **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 (89). The RFX1, Sp1, C/EBP, HNF3 and TBP recognition sequences are underlined (89). 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, HNF4α (76), RXRα/PPARα (76), RXRα/FXRα (71), LRH1 (52) 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.
1.1.5 **HBV Transgenic Mice**

Analysis of HBV biosynthesis in non-hepatoma cells has demonstrated that nuclear receptors are the only transcription factors capable of supporting HBV 3.5-kb pregenomic RNA synthesis and viral replication (89). 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 (29). Unlike natural infection, the HBV transgenic mouse model can only be used to evaluate the quantitative effect of a gene product or a treatment on a single replication cycle. The HBV transgenic mouse is not an infection system.

Based on our cell culture studies and using the HBV transgenic mouse model of chronic HBV infection, the role of PPARα in HBV biosynthesis was investigated *in vivo* (28). This analysis demonstrated that PPARα did not play a role in determining the level of viral biosynthesis in the liver under normal physiological conditions. However, treatment of HBV transgenic mice with peroxisome proliferators enhanced viral transcription and replication in a PPARα-dependent manner indicating that this nuclear receptor can activate viral biosynthesis under certain circumstances (28).

The second nuclear receptor examined in the HBV transgenic mouse model of chronic infection was HNF4α (51). HNF4α is a transcription factor that is essential for the viability of the mouse (14). However, utilizing the conditional tissue-specific deletion of HNF4α in the neonatal liver, it was possible to determine the role of this nuclear receptor in HBV biosynthesis during early...
postnatal development (51). Loss of HNF4α expression resulted in the absence of HBV transcription and replication in the livers of the HBV transgenic mice demonstrating that HNF4α is essential for viral biosynthesis in vivo. This finding supports the analysis in non-hepatoma cells where HNF4α binding to the proximal recognition sequence within the nucleocapsid promoter is essential for HBV 3.5kb pregenomic RNA synthesis and viral replication (89).

Although HNF4α is essential for viral biosynthesis in early neonatal development in the HBV transgenic mouse, it is still not established if the effects of HNF4α in vivo are direct or indirect. However, it is apparent that the effect of HNF4α occurs at the level of viral transcription because the loss of this nuclear receptor is associated with a corresponding decrease in HBV transcription (51). If the effect of HNF4α is not directly on viral transcription, it most likely affects this process by altering the activities of additional transcription factors and/or their associated coactivators that can support HBV biosynthesis which, like HNF4α, are involved in the complex transcription factor regulatory network required to produce and maintain the differentiated hepatic phenotype (42). As loss of PPARα does not affect HBV biosynthesis under normal physiological conditions, it seems most likely that additional nuclear receptors may represent the alternative liver-enriched transcription factors that might direct HBV transcription and replication.
1.1.6 Role of other nuclear receptors in HBV biosynthesis

Additional nuclear receptors have been identified that bind to and regulate nucleocapsid promoter activity in hepatoma cells. Notably, FXRα was found to modulate HBV nucleocapsid promter activity (71). Two RXRα/FXRα binding sites have been identified within the nucleocapsid promoter. Therefore, one aim of these studies was to determine the ability of RXRα/FXRα to support HBV 3.5-kb pregenomic RNA synthesis and viral replication in non-hepatoma cells. In addition, since the ligand for FXRα is bile acids, another overall aim of these studies is to determine the role of bile acids in HBV biosynthesis.

1.2 Bile Acids

1.2.1 Metabolic function of bile acids

The liver plays a major role in the digestion of lipids through the synthesis of bile acids that are subsequently secreted and transported to the duodenum (15). Bile acids are made from cholesterol, and cytochrome P450 7A1 (CYP7A1) is the rate-limiting enzyme in bile acid synthesis. Bile acid conjugation decreases toxicity and increases solubility for secretion into bile. Once bile acids are conjugated they are secreted into bile by the bile salt export pump (BSEP) and stored in the gallbladder, which upon contraction gives postprandial secretion of bile into the intestine. Once in the gut lumen, bile acids are physiological detergents that aid in the aqueous solubilization of lipophilic molecules such as lipids, nutrients and vitamins. Bile acids also function as signaling molecules (15).
1.2.2 Bile acids, FXRα and Small Heterodimer Partner (SHP)

Bile acids are the natural ligands for the nuclear receptor, FXRα. Both free and conjugated bile acids bind to the FXRα ligand binding domain. FXRα heterodimerizes with RXRα and the heterodimer stimulates gene transcription of FXRα target genes by binding to the inverted repeat of AGGTCA-like sequences with one nucleotide spacing (IR1) in the promoter of the target gene. FXRα regulation has many roles in the liver including promoting resolution of liver fibrosis, lowering blood glucose and lipid levels, and enhancing insulin sensitivity in diabetic mice (21, 45, 57, 65, 101, 107). FXRα regulates endogenous bile acid synthesis in the liver, in part, through the transcriptional activation of the small heterodimer partner (SHP) gene (Fig 3) (20, 26, 56, 58, 69). SHP is also a member of the nuclear receptor family of transcription factors, but it is atypical in that it lacks a DNA binding domain and generally represses gene expression of various transcription factors including other nuclear receptors (Fig 3) (83, 102). SHP is referred to as a corepressor because it acts to repress multiple nuclear receptors and it represses these nuclear receptors in two ways. SHP both competes with coactivators for binding to nuclear receptors and functions directly as a transcriptional repressor (47, 48). Indeed, SHP decreases the rate limiting step in bile acid synthesis by inhibiting liver X receptor- (LXR-) and liver receptor homolog 1-(LRH1-) mediated expression of the CYP7A1 gene (Fig 2) (26, 56). Additionally, SHP inhibits its own expression in a negative feedback loop aimed at maintaining appropriate bile acid homeostasis within the liver (Fig 3) (26, 56). SHP has various roles in glucose homeostasis, cholesterol and bile acid
metabolism, and lipogenesis (4, 9). Cell culture studies have revealed the ability of SHP to inhibit HBV biosynthesis in hepatoma cells (67). The induction of SHP may be a feasible approach to inhibit HBV biosynthesis in human infection, especially if HBV is regulated by multiple nuclear receptors and the contribution of FXRα is limited. Consequently, the effect of bile acid treatment on viral biosynthesis in SHP-expressing and SHP-null HBV transgenic mice is of great interest to determine the relative importance of FXRα and SHP for HBV transcription and replication \textit{in vivo}.

![Diagram of regulatory network in the liver](image)

Figure 3. \textbf{Components of the regulatory network in the liver governing bile acid synthesis and their potential effects on nuclear receptor-mediated HBV biosynthesis.} Bile acids are the ligands for FXR and increase its transcriptional activity (58, 69). FXR activates SHP gene expression whereas SHP generally represses nuclear receptor-mediated transcription including the activities of HNF4, RXR/FXR, RXR/PPAR, LRH1 and ERR (4, 26, 56). FXR directly regulates bile salt export pump (BSEP) gene expression (1). Cytochrome P450 7A1 (CYP7A1) gene expression is regulated by multiple nuclear receptors and encodes the enzyme mediating the rate limiting step in bile acid biosynthesis (26, 36, 56). Nuclear receptors activate HBV RNA and DNA replication intermediate synthesis (89).
1.3 **Fox A/HNF3**

1.3.1 **FoxA discovery and overview**

Hepatocyte nuclear factor 3 (HNF3) genes were initially cloned based on their ability to bind regulatory regions of the liver-enriched α1-antitrypsin and transthyretin genes (31, 44). Afterwards, the nomenclature of this gene family has been revised to FoxA because HNF3 encodes a winged helix protein (31, 44). FoxA factors are monomeric forkhead box winged helix transcription factors with a centrally located 100 amino acid DNA-binding domain (22). FoxA proteins have been found to play many important roles with close to 500 genes discovered to have FoxA2 binding sites in either their promoters or enhancers. Overall FoxA3 is less closely related than FoxA1 and FoxA2. However, all FoxA forkhead box protein sequences are 95% identical and each has sequences for nuclear localization. One significant difference is that FoxA2, but not FoxA1 or FoxA3, contains an AKT/PKB phosphorylation site at the N terminus of the forkhead domain. There is currently a controversial model of insulin-mediated AKT signaling leading to the phosphorylation of FoxA2 and its subsequent nuclear exclusion. This model is controversial because others have found FoxA2 protein consistently in the nucleus regardless of metabolic state (22).

1.3.2 **Role of FoxA in organ development**

FoxA2 is the first of the three FoxA genes to be activated with mRNA and protein gene expression at embryonic day 6.5. FoxA1 is activated shortly after at embryonic day 7 and FoxA3 gene expression is first seen on embryonic day 10.5
(44). Through the development of knock out mice, FoxA2 was discovered to be required for the formation of the node and notochord since embryos lacking Foxa2 expression lack foregut endoderm and notochord, an essential source of sonic hedgehog signaling to pattern the neural tube. Furthermore, FoxA1 and FoxA2 cooperate for normal development of endoderm-derived organs such as the liver, pancreas, lungs and prostate (22). When FoxA1 and FoxA2 genes are deleted, hepatic specification is completely blocked. The FoxA1/FoxA2-deficient mouse is the only currently known model of a “liver-less” vertebrate (44). Also deletion of both FoxA1 and FoxA2, but not deletion of either factor alone, blocks branching morphogenesis in the lung (39).

1.3.3 **FoxA proteins as pioneer factors**

FoxA factors are capable of promoting gene activation by altering chromatin structure through displacement of linker histone H1. FoxA proteins have been identified as pioneer factors. Pioneer factors trigger transcriptional competency through initial chromatin decompaction. FoxA proteins can replace linker histones, directly affecting chromatin structure (39). Binding of FoxA proteins to promoters and enhancers enable chromatin access for other tissue-specific transcription factors (22).

1.3.4 **FoxA as binding partners of nuclear receptors**

FoxA proteins can also function to facilitate the binding of nuclear hormone receptors to their targets in multiple organ systems (22, 30). FoxA
factors have been shown to play a major role in gene activation by the glucocorticoid, androgen and estrogen receptors (39). Glucocorticoid receptor (GR) binding to its targets in vivo was demonstrated by chomatin immunoprecipitation (ChIP) to be FoxA2-dependent and is one example of nuclear receptor binding being FoxA-dependent (105). Conditional gene ablation of FoxA2 in the liver established that this factor regulates the expression of the fasting-induced genes tyrosine aminotransferase, phosphoenolpyruvate carboxykinase 1 (Pck1) and insulin-like growth factor-binding protein 1 (Igfbp1). This study concluded that FoxA2 is required to facilitate chromatin access of the GR for maximal induction of target genes during fasting.

1.3.5 FoxA and bile acid regulation

FoxA2 regulates multiple genes involved in bile acid metabolism (6, 40). One study speculated that the presence of FoxA2 binding sites near bile-acid response elements targeted by FXR signified that FoxA is required to activate a set of genes critical for the hepatic response to cholic acid (CA). The authors found that the absence of FoxA2 leads to intrahepatic cholestasis and liver injury in mice fed a diet supplemented with CA, a natural ligand for FXR (5, 6). This study deleted FoxA2 in hepatocytes in mice using the Cre-lox system and found decreased transcription of genes encoding bile acid transporters, resulting in intrahepatic cholestasis. FoxA2-deficient mice have normal bile acid concentration in sera when on a normal diet, but FoxA2-deficient mice have markedly increased hepatic and sera bile acid content when on a cholic acid diet
(compared to wild type mice). Interestingly, this study noted that the FoxA2 protein is virtually undetectable in pediatric patients with primary sclerosing cholangitis, as well as in those patients with biliary atresia. By mRNA and protein studies, it appears that mice with hepatocytes deficient in FoxA2 had complex alterations in levels of transporters and modifying enzymes for bile acids leading to increased intrahepatic bile acid levels. A cholic acid diet that was well tolerated in the control mice lead to increased liver injury in the FoxA2 deficient mice. Many of the genes encoding bile acid enzymes and transporters were determined to be direct targets of FoxA2.

Another paper characterized Foxa1\textsuperscript{loxP/loxP} Foxa2\textsuperscript{loxP/loxP} Alf-pCre mutant mice and found hyperplasia of biliary tree and abnormal bile duct formation (53). Two conditional (loxP-flanked) alleles were employed to simultaneously ablate both Foxa1 and Foxa2 during fetal liver development using an albumin -α-fetoprotein Cre transgene. The authors determined that abnormal liver phenotype was due, at least in part, to activation of interleukin 6 (IL-6) expression, a proliferative signal for cholangiocytes. GR is a negative regulator of IL-6 and in the absence of FoxA1 and FoxA2, GR failed to bind to the IL-6 promoter. Mutant mice did not show any significant changes in blood biochemistry but collagen fibers were found in the portal tracts without any significant change in transforming growth factor β (TGF β) or α smooth muscle actin (αSMA) mRNA levels. One difference between wild type and mutant mice was alpha fetoprotein (AFP) mRNA which was found to be 10 fold higher in Fox-A deficient livers. The authors also stated that proliferative markers showed that while the
proliferative rate of hepatocytes was unaffected by deficiency of FoxA1 and FoxA2, proliferation of cholangiocytes was dramatically increased. Mutant mice were found to have an abnormal and expanded biliary tree and this bile duct hyperplasia was caused by increased proliferation of biliary epithelial cells. This proliferation was the result of cis-regulation of the IL-6 promoter. Similar to the previously mentioned FoxA dependent GR binding to the promoter of the Pck1 gene, the occupancy of glucocorticoid receptor on the IL-6 promoter was determined to be FoxA-dependent. Thus, when FoxA is deficient there is decreased GR binding to the IL-6 promoter and IL-6 transcription is derepressed as GR is replaced by NF-κB, a strong activator of IL-6 expression. The resulting increase in IL-6 expression leads to the cholangiocyte hyperproliferation.

1.3.6 Role of FoxA in regulating HBV biosynthesis in cell culture

The final major objective of this study is to determine the role of FoxA in HBV transcription and replication in vivo. It has been shown previously that FoxA increases transcription from the nucleocapsid promoter to approximately the same extent as nuclear receptors using reporter gene constructs and transient transfection analysis (75, 77). Consequently, it might have been expected that FoxA/HNF3 would have stimulated HBV 3.5kb RNA synthesis and viral replication with similar efficiency to the nuclear receptors under these same transfection conditions. However, this is not the case. This suggests that measuring transcription from reporter gene constructs is not equivalent to measuring transcription from greater-than-genome length HBV constructs capable
of supporting viral replication. In fact, FoxA/HNF3 inhibited nuclear receptor-mediated viral replication by a mechanism that involves a specific reduction in the level of the HBV 3.5kb pregenomic RNA (89).

However, recently our laboratory has demonstrated that FoxA can support HBV biosynthesis in a non-hepatoma HBV replication system when the specific coactivator, PGC1α, is cotransfected with the FoxA expression vector (Ondracek and McLachlan, personal communication). Additionally, this system has been utilized to demonstrate that the different FoxA transcription factors preferentially utilize distinct coactivators to support HBV 3.5kb pregenomic RNA synthesis and viral replication (Ondracek and McLachlan, personal communication). Together, these observations suggest that FoxA in combination with the necessary coactivator can robustly support viral biosynthesis. Hence the reason FoxA fails to support HBV biosynthesis in nonhepatoma cells appears to be because there is a deficiency in the required activity level(s) of the appropriate coactivator(s).

Indeed, it seems likely that the reason FoxA can inhibit nuclear receptor-mediated biosynthesis in nonhepatoma cells is due to its capacity to mediate the formation of non-functional transcriptional pre-initiation complexes at intragenic viral promoters (i.e. the PS1, S, X and/or C gene promoter). This presumably results from the lack of the necessary coactivators in these cells.

1.3.7 HNF4α regulation of FoxA

As mentioned in section 1.1.5, the loss of HNF4α expression resulted in the absence of HBV transcription and replication in the livers of the HBV
transgenic mice demonstrating that HNF4α is essential for viral biosynthesis in vivo, and if the effect of HNF4α is not directly on viral transcription, it most likely affects this process by altering the activities of additional factors that can support HBV biosynthesis. The loss of HNF4α has been previously reported to lead to decreased FoxA2 expression (32, 70). Therefore, it is possible there are decreased FoxA levels in the HNF4α-deficient HBV transgenic mice, and, if so, perhaps this decrease in FoxA could be an indirect mechanism by which loss of HNF4α in development leads to an absence of HBV biosynthesis in the HBV transgenic mouse.

1.4 Summary

First, this study will assess the transcriptional regulation of HBV biosynthesis in non-hepatoma cells by a subset of nuclear receptors, including assessment of the role of the nucleocapsid promoter sequence in this transcriptional regulation. The inhibitory role of SHP will also be assessed in cell culture. In addition, the effect of bile acid treatment on HBV biosynthesis will be investigated in SHP-expressing and SHP-null HBV transgenic mouse. Furthermore, this study will determine whether FoxA expression is down-regulated in the liver-specific HNF4α-deficient HBV transgenic mice with absent HBV biosynthesis, and therefore a possible indirect mechanism for inhibition of HBV biosynthesis in these mice. Lastly, this study will determine the role of FoxA in HBV biosynthesis in vivo using FoxA-deficient HBV transgenic mice.
2. METHODS

2.1 HBV biosynthesis in cell culture

2.1.1 Plasmid construction

The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (81). 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 (19). The HBV DNA (4.1kbp) construct that contains 1.3 copies of the HBV genome includes the viral sequence from nucleotide coordinates 1062 to 3182 plus 1 to 1990 (28). This plasmid was constructed by cloning the Nsil/BglIII HBV DNA fragment (nucleotide coordinates 1062 to 1990) into pUC13, generating pHBV(1062-1990). Subsequently, a complete copy of the 3.2kbp viral genome linearized at the Ncol site (nucleotide coordinates 1375 to 3182 plus 1 to 1374) was cloned into the unique Ncol site (HBV nucleotide coordinate 1374) of pHBV(1062-1990), generating the HBV DNA (4.1kbp) construct.

The pCMVHNF4α, pRS-hRXRα, pCMVPPARα-G, pCMV-rFXRα, pCMX-mLRH1, pSG5-mERRα, pcDNA3-2xFLAG-mERRβ and pSV5- mERRγ vectors express HNF4α, RXR, PPARα-G, FXRα, LRH1, ERRα, ERRβ and ERRγ polypeptides from the rat HNF4α, human RXRα, mouse PPARα-G, rat FXRα, mouse LRH1, mouse ERRα, mouse ERRβ and mouse ERRγ 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) (7, 13, 41, 56, 60, 66). The PPARα- G polypeptide
contains a mutation in the PPARα 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 containing promoter (66). The pcDNA3-HA-hPGC1α and pCMXSHP vectors express PGC1α and SHP polypeptides from the human PGC1α and mouse SHP cDNAs, respectively, using the CMV immediate-early promoter (pcDNA3 and pCMX) (41, 56).

2.1.2 Cells and transfection

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% CO₂/air. Transfections for viral RNA and DNA analysis were performed as previously described using 10 cm plates, containing approximately 1 X 10⁶ cells (63). Cells were split one day prior to transfection. Two hours prior to transfection the media was changed to transfection media (DMEM without FBS). DNA was dispensed into eppendorf tubes using 10 mM Tris hydrochloride, pH8.0, 1 mM EDTA to bring volume up to 40ul. In 293T cells, the transfected DNA mixture was composed of 5 mg of HBV DNA (4.1kbp) plus 1.5 mg of the nuclear receptor expression vectors, pCMVHNF4α, pRS-hRXRα, pCMVPPARα-G, pCMV-rFXRα, pCMX-mLRH1, pSG5-mERRα, pcDNA3-2xFLAG-mERRβ and pSV5- mERRγ, and various amounts of the pcDNA3-HA-hPGC1α and pCMXSHP expression vectors. In tissue culture hood, 463ul of
CaCl$_2$ H20 mix was added to each eppendorf tube and 500ul sterile 2xHeBs buffer was added to a sterile falcon 2063 tube for each plate of cells to be transfected. Then the contents of each eppendorf tube was added to the contents of the respective falcon 2063 tube – slowly and while vortexing. Samples were left to incubate for 30 minutes at room temperature in the hood. Then the approximately 1 ml mixture was added to its respective plate of cells and plates were swirled to mix. Plates were then put back in the incubator and 4 hours later the glycerol shock was performed by aspirating the media and then adding 2ml of glycerol solution (10mM Hepes pH 7.6, 140mM NaCl, 15% glycerol, filtered through .2um filter to sterilize) to each plate. Plates were incubated for 2 minutes at 37°C. After incubation the solution was aspirated and cells were washed with 4ml of DMEM media without serum. After aspirating the wash media, 10ml of normal growth media was added and then ligands were added, if necessary. 10ul of all-trans retinoic acid, clofibric acid, and chenodeoxycholic acid at 1mM, 1mM and 100mM, respectively, were used to activate the nuclear receptors, RXR$\alpha$, PPAR$\alpha$ and FXR$\alpha$ (89). Ligand stocks were kept in DMSO. DNA and RNA isolation was performed 3 days post transfection.

2.1.3 Characterization of HBV transcripts and viral replication from cell culture

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 with minor modifications (87). For RNA isolation 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 (16). 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-isooamyl alcohol (49:1). Samples vortexed and incubated on ice for 15 minutes. After centrifugation for 30 min. at 3,000 rpm in a Sorval RT6000, the aqueous layer was precipitated with 1.8 ml of isopropanol for 15 minutes at -70°C or greater than one hour at -20°C. Then samples centrifuged 20 minutes at 10,000 rpm in HB-4 rotor and 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 for 15 minutes at -70°C or overnight at -20°C. After centrifugation for 20 min. at 14,000 rpm in an Eppendorf 5417C microcentrifuge at 4°C, the precipitate was washed with 70% ethanol and then 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 ml 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 ug/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 tris-saturated phenol, and extracted twice with ether. Then 32ul NH₄OAc was added along with 2 volumes of ethanol and samples were precipitated for 15 minutes at -70°C or overnight at -20°C. Samples were centrifuged for 20 minutes at 14,000 rpm in an Eppendorf 5417C microcentrifuge at 4°C and pellet was washed with 70% ethanol. Then samples were resuspended in 100 µl of 10 mM Tris hydrochloride, pH8.0, 1 mM EDTA. RNA (Northern) and DNA (Southern) filter hybridization analysis were performed using 10 µg of total cellular RNA and 30 ul of viral DNA replication intermediates, respectively, as described (81). Filters were probed with ³²P-labeled HBVayw genomic DNA to detect HBV sequences and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA to detect the GAPDH transcript used as an internal control. Filter hybridization analyses were quantified by phosphorimaging using a Packard Cyclone Storage Phosphor System.

2.2 **HBV biosynthesis in HBV transgenic mice**

2.2.1 **Transgenic mice: generation and genotyping**

The production and characterization of the HBV transgenic mouse lineage 1.3.32 has been described (29). These HBV transgenic mice contain a single copy of the terminally redundant, 1.3-genome length copy of the HBVayw genome integrated into the mouse chromosomal DNA. High levels of HBV replication occur in the livers of these mice. The mice used in the breeding experiments were homozygous for the HBV transgene and were maintained on the SV129 genetic
background (46). The SHP-null mice were a generous gift from David D. Moore, Baylor College of Medicine, and the production and characterization of the SHP-null mice has been described (94, 95). These mice do not express SHP, which contributes to bile acid and cholesterol homeostasis (94, 95). The mice used in the breeding experiments were homozygous null for SHP and maintained on the C57B1/129SV hybrid genetic background (94, 95).

SHP-null HBV transgenic mice were generated by mating the HBV transgenic mice with the SHP-null (-/-) mice. The resulting SHP heterozygous (+/-) HBV transgenic F1 mice were subsequently mated with the SHP-null (-/-) mice and the F2 mice were screened for the HBV transgene and SHP-null allele by polymerase chain reaction (PCR) analysis of tail DNA. Tail DNA was prepared by incubating 1 cm of tail in 500 ml of 100 mM Tris hydrochloride (pH 8.0), 200 mM NaCl, 5 mM EDTA, 0.2% (wt/vol) SDS containing 100 mg/ml Proteinase K for 16 to 20 hours at 55°C. Samples were centrifuged at 14,000 rpm in an Eppendorf 5417C microcentrifuge for 10 minutes and the supernatant was precipitated with 500 ml of isopropanol. DNA transferred to 80% ethanol and then pelleted by centrifugation at 14,000 rpm in an Eppendorf 5417C microcentrifuge for 10 minutes and subsequently dissolved in 100 ml of 5 mM Tris hydrochloride (pH 8.0), 1 mM EDTA. The HBV transgene was identified by PCR analysis using the oligonucleotides, 5’-TCGATACTGAAACCTTTACCCC GTTGCCCCG-3’ (oligo XpHNF4-1, HBV coordinates 1133 to 1159) and 5’-TCGAATTGCTGAGAGTCCAAGAGTCCTCTT3’ (oligo CpHNF4-2, HBV coordinates 1683 to 1658), and 1 ul of tail DNA. A PCR product of 551 bp
indicated the presence of the HBV transgene. The SHP-expressing and null alleles were identified by PCR analysis using the oligonucleotides, 5' -
CTCTGCAGGTCGTCCGACTATTCTG-3' (Exon-1F) and 5'-CCTCGAAGGT
CACAGCATCCTG-3' (Exon-1B) located in the deleted first exon of the SHP gene coding region, and 5'-CTAGCTAGAGGATCCCC GGGTACC-3' (Gal-PCR 5') and 5'-AATTCGCGTCTGGCCTTCCTGTAG-3' (Gal-PCR 3') located in the β-gal cassette, respectively, and 1 ul of tail DNA (94). A PCR product of 296 bp indicated the SHP-expressing allele whereas a PCR product of 500 bp indicated the SHP null allele. The samples were subjected to 42 amplification cycles involving denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension from the primers at 72°C for 2 minutes. The reaction conditions used were as described by the manufacturer (Genscript) and contained 2.5 units of Taq DNA polymerase.

Floxed FoxA1, floxed FoxA2 and FoxA3-null mice were a generous gift from Dr. Klaus H. Kaestner, University of Pennsylvania School of Medicine (37, 86). Several strains of FoxA-deficient HBV transgenic mice have been developed. These include (i) liver-specific FoxA1-null, (ii) liver-specific FoxA2-null, (iii) FoxA3-null, (iv) liver-specific FoxA1+FoxA2-null and (v) liver-specific FoxA1+FoxA2-null plus FoxA3(+/−) HBV transgenic mice. Liver-specific loss of FoxA1, FoxA2 and FoxA3 failed to generate viable mice for this study. Mice with the AlbCre transgene from Jackson labs, shown to be very efficient for performing liver-specific gene knockouts, were used in this study. The AlbCre transgene was identified by PCR analysis using the oligonucleotides, 5’-CCAGC
TAAACATGCTTCATCGTCG-3’ and 5’-ATTCTCCACCGTCAGTACG
TGAG-3’, and 1 ul of tail DNA. A PCR product of 300 bp indicated a cre
genotype of (+/-) or (+/+). FoxA1 genotype was identified by PCR analysis using
the oligonucleotides, 5’-CTGTGGATTATGTTTCCTGATC-3’ and 5’-GTGTCAG
GATGCCTATCTGGT-3’, and 1 ul of tail DNA. A PCR product of 290 bp
indicated the wild-type band and a 480 bp band indicated a floxed band. FoxA2
genotype was identified by PCR analysis using the oligonucleotides, 5’-CCCCTG
AGTTGGCGGTGGT-3’ and 5’-TTGCTCACGGAAGAGTAGCC-3’, and 1 ul of
tail DNA. A PCR product of 290 bp indicated the wild-type band and a 450 bp
band indicated a floxed band. FoxA3 genotype was identified by PCR analysis
using the oligonucleotides, FoxA3 F 5’-GGCAGTGCTTCCCGGTATGTA-3’,
Foxl1-7 5’-GGGAAGAGGTCCATGATCCAT-3’ and LacZ-3 5’-CAAAGGC
GCCATTCGCCATTCA-3’, and 1 ul of tail DNA. A PCR product of 196 bp
indicated the wild-type band and a 290 bp band indicated a floxed band.

HBV transgenic mice were fed normal rodent chow (control) or rodent
chow containing 1% (wt/wt) cholic acid (CA) for 7 days (94). Water was
available ad libitum. Mice were sacrificed and liver tissue was frozen in liquid
nitrogen and stored at –70°C prior to DNA and RNA extraction.

2.2.2 Liver HBV DNA and RNA analysis

Total DNA and RNA were isolated from liver of HBV transgenic mice as
described (16, 81). DNA isolation was performed by addition of 5ml of DNA
lysis Buffer (10mM Tris-HCl pH8.0, 10mM EDTA pH 8.0, 10mM NaCl, 0.5%
SDS) to a 15ml conical tube and processed one sample at a time by placing frozen tissue in the buffer and immediately pulverized at full speed with homogenizer until chunks are gone and solution appeared homogenous. Homogenizer probe was washed in between samples with 10% SDS solution and rinsed with water and wiped dry. After all samples were processed, 25ul of 20 mg/ml Proteinase K (final concentration of 100 ug/ml) was added, mixed gently and samples were incubated overnight at 37°C on a rocking platform. The next day, samples were transferred to Falcon 2059 tubes and 5ml of tris-saturated phenol/chloroform/isoamyl alcohol (50:48:2) was added and mixed well by vortexing. Then samples were centrifuged for 10 minutes at 10,000 rpm in an HB-4 rotor at room temperature. This centrifugation was repeated until the aqueous phase was clear. The aqueous phase was placed into a new 2059 tube and 1 volume of chloroform:isoamyl alcohol (24:1) was added and vortexed. Samples were centrifuged for 10 minutes at 3,000 rpm in a Sorval RT6000 at room temperature. The aqueous phase was transferred to a new Falcon 2059 tube and 1/10 volume of 3M NaOAc pH5.2 was added and mixed gently. Then 2.5 volumes of ice-cold 100% ethanol was added, and samples were mixed and precipitate at -70°C for greater than one hour. Samples were centrifuged for 10 minutes at 3,000 rpm in a Sorval RT6000 at 4°C. Pellet was washed with 70% ethanol and dissolved in 1ml of 10 mM Tris hydrochloride, pH8.0, 1 mM EDTA. Samples were RNase treated by adding 1ul of 10mg/ml RNase A per 1ml of DNA sample and rocked for 3 hours at 37°C. Samples were stored at -70°C.
RNA isolation was performed by addition of 5ml of denaturing solution (25 mM sodium citrate, pH 7.0, 4 M guanidinium isothiocyanate, 0.5% (v/v) sarcosyl, 0.1 M 2-mercaptoethanol) to a 15ml conical tube and processed one sample at a time by placing frozen tissue in the buffer and immediately pulverized at full speed with homogenizer until the solution appeared homogenous. The homogenizer probe was washed in between samples with dilute SDS solution and rinsed with water and wiped dry. Once samples were processed, 500ul of 2M sodium acetate, pH 4.0 was added to each sample and samples were vortexed. Then 5 ml of water-saturated phenol plus 2 ml of chloroform-isoamyl alcohol (49:1) was added to each sample. Samples were vortexed, transferred to a Falcon 2059 tube and incubated on ice for 10 minutes. Then samples were centrifuged for 20 minutes at 8,000 rpm in HB-4 rotor at 4°C. The aqueous phase was placed into a new 2059 tube and 4ml of cold isopropanol was added and vortexed. Samples were precipitate at -70°C for greater than one hour and then thawed samples were centrifuged for 10 minutes at 3,000 rpm in a Sorval RT6000 at 4°C. Tubes were inverted to drain liquid and a Kimwipe was used to wick away any residual alcohol from the pellet. Pellet was resuspended in 2ml denaturing solution and vortexed until the pellet completely dissolved. 2ml of 100% ethanol was added and samples were vortexed and precipitated at -70°C for greater than one hour. Thawed samples were centrifuged for 5 minutes at 3,000 rpm in a Sorval RT6000 at 4°C. Tubes were inverted to drain the liquid, the pellet was washed with 70% ethanol, and a Kimwipe was used to wick away any residual alcohol from the pellet. The pellet was resuspended 0.3 ml of 10 mM Tris hydrochloride, pH 8.0, 5
mM EDTA, 0.1% (w/v) sodium lauryl sulfate. Samples were transferred to an eppendorf tube and precipitated with 45 ml of 2 M sodium acetate plus 0.7 ml of ethanol. Samples were vortexed and stored at -70°C.

DNA (Southern) filter hybridization analyses were performed using 20 ug of HindIII digested DNA (81). Filters were probed with \(^{32}\)P-labeled HBV\textit{ayw} genomic DNA to detect HBV sequences (23). RNA (Northern) filter hybridization analyses were performed using 10 ug of total cellular RNA as described (81). Filters were probed with \(^{32}\)P-labeled HBV\textit{ayw} genomic DNA to detect HBV sequences and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA to detect the GAPDH transcript used as an internal control. Filter hybridization analyses were quantified by phosphorimaging using a Packard Cyclone Storage Phosphor System.

2.2.3 RT-qPCR

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to measure the level of HBV 3.5kb transcripts in mouse liver RNA and liver RNA levels of all the other genes listed in Table 1. After DNase I treatment, 1 ug of RNA was used for cDNA synthesis using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA), followed by real-time PCR quantification using SYBR Green and an Applied Biosystems 7300 real-time thermocycler (Applied Biosystems). Thermal cycling consisted of an initial denaturation step for 10 min at 95°C followed by 40 cycles of denaturation (15 sec at 95°C) and annealing/extension (1 min at 60°C). The relative RNA
expression levels of the gene of interest were estimated using the ΔΔCt method with normalization to mouse GAPDH RNA (54, 68, 80). The PCR primers used to measure HBV 3.5kb transcripts were 5’-GCCCCTATCTATCAACACTTC CG-3’ (HBV 3.5kb RNA sense primer, coordinates 2311 to 2335) and 5’-TTCGTCGACGGCGAGGG-3’ (HBV 3.5kb RNA antisense primer, coordinates 2401 to 2382). All other RT-qPCR primers used are listed in Table 1.
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<th>Antisense primer</th>
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<td>TNFa</td>
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2.2.4 Serum HBV E antigen and ALT analysis

HBeAg analysis was performed using 2 ul of mouse serum and the HBe enzyme linked immunosorbent assay as described by the manufacturer (Epitope Diagnostics). The positive control was diluted 1:5 in PBS and then used for a standard curve. 48ul PBS plus 2ul of sera was added to the sample wells. 50ul of enzyme conjugated antibody was added to every well except the blank well and the plate was incubated at 37°C for 30 minutes. The wash buffer included in the kit was diluted 1:20 to give enough wash buffer for 350ul per well for five washes. After the incubation, the plate is washed five times per well using 350ul wash buffer per well each time. Each wash consists of adding the wash buffer to every well and then inverting the plate to get rid of the liquid. After washing the plate, 50ul of substrate solution A was added to every well and then 50ul of solution B was added to every well. The plate was then covered and incubated at 37°C for 15 minutes. After incubation, 50ul of stop solution was added to every well and the color intensity of the solution in each well was measured using a microwell reader at 450nm. The level of antigen was determined in the linear range of the assay.

Alanine aminotransferase (ALT) activity was determined using 10 ul of mouse serum as described by the manufacturer (Genzyme ALT assay 318-10). The level of ALT was determined in the linear range of the assay using an ALT positive control (Cayman Chemical item number 700265). 96-well plate template was labeled (including a blank well, well with 10ul PBS plus ALT mix, wells with positive control plus ALT mix and sample wells of 10ul sera plus ALT
mix). The positive control was reconstituted with 400ul of diluted ALT Assay Buffer (Cayman Chemical item number 700261) to yield a 1.0 IU/ml solution and aliquoted into eppendorf tubes to keep at -80°C for up to one month. At the time of the assay, one eppendorf tube of the positive control was thawed for the generation of the calibration curve. The positive control and samples were added to the 96-well plate. 140ul R1 reagent and 35ul R2 reagent per well were mixed in a Falcon 2059 tube. 175ul ALT mix was quickly added to every well except the blank well and the plate was read immediately. Plates were assayed by measuring absorbance at 340nm every 2 minutes for 10 minutes. The rate of decrease in absorbance at 340nm due to the oxidation of NADH to NAD, is directly proportional to the ALT activity. The slope from the positive control was used to calculate the ALT level for each sample.

2.3 **Immunohistochemical staining**

Tissues were placed in formalin, fixed overnight and then given to the Research Histology and Tissue Imaging Core at University of Illinois at Chicago to perform immunohistochemical staining. Tissues were embedded in paraffin, sectioned and stained. HBcAg staining was done with rabbit anti-HBcAg (Dako, N155630). Masson trichrome staining was performed to assess fibrosis.

2.4 **Adenovirus vector**

HEK293 cells were infected at 60-80% confluency. Media and adenovirus vector, 2.5ml serum-free DMEM with 2.5ul virus, with a titer of approximately
1x10^{10} pfu/ml, per 10 cm plate, were mixed and 2.5 ml of mix was added to each plate. When amplifying for CsCl purification 10 10 cm plates were used. Plates were swirled to mix and put back in incubator for 1 hour with rotation every 10 minutes. Then 7.5 ml of regular media (DMEM with 10% serum) was added to each plate. Cells started detaching within 24-48 hours. Cells were harvested when 80% of the cells were floating. Cells and media were collected in 50 ml conical tubes and centrifuged cells at 1000 rpm for 5 min at 4°C. Supernatant was discarded and cell pellet from 5 10 cm plates was resuspended in 4 ml sterile PBS and mixed by pipeting up and down. Cells were then centrifuged again at 1000 rpm for 5 min at 4°C and cell pellet from 5 10 cm plates was resuspended in 4 ml sterile PBS. The cells in PBS underwent 3 cycles of freezing and thawing: freezing in dry ice/ethanol mix, thawing in 37°C water bath, and very gently vortexing after each thaw. Cells were transferred to 2059 falcon tubes and viral lysate was centrifuged in a Sorvall refrigerated centrifuge at 7000xg (HS-4 rotor at 6000 rpm) at 4°C for 5 minutes. While avoiding the cell pellet, 8 ml of cleared viral supernatant was transferred to a 50 ml conical tube containing 4.4 g of cesium chloride (CsCl) and mixed well by vortexing. The CsCl solution (~10 ml, density of 1.35 g/ml) was transferred to a 12 ml polyallomer tube for a SW 41 Ti rotor. The CsCl solution was overlaid with mineral oil to fill the tube. Samples were centrifuged in a Beckman ultracentrifuge with an SW 41 Ti rotor for 18 to 24 hour at 176,000xg (SW 41 Ti rotor at 32,000 rpm) at 10°C. Tubes were removed from ultracentrifuge. The position of the virus band, which appears as a narrow opaque white band ~1 to 2 cm below the mineral oil interface, was noted, and the
virus fraction (~.5 to 1ml) was collected with a 3ml syringe and 18 gauge needle by puncturing the side of the tube under the band to extract it into the syringe. The virus fraction was then mixed with an equal volume of 2x filtered storage buffer (10mM Tris-Cl, pH 8.0, 100mM NaCl, 50% glycerol). Virus stocks (of approximately 1x10^{12} pfu/ml) were stored at -80°C. The day prior to injection into mice the virus stock was transferred into a dialysis cassette (Pierce #66110, 3.5K MWCO) and dialyzed in PBS plus 10% glycerol for 1-2 hours. The buffer was changed to fresh PBS plus 10% glycerol after first dialysis and subsequently dialyzed overnight. Adenovirus vector was removed from the dialysis cassette, filtered through a 22um filter and 100ul was injected by retro-orbital injection into mice.
3. ROLE OF FXR AND SHP IN HBV BIOSYNTHESIS

3.1 HBV biosynthesis in cell culture

3.1.1 Multiple nuclear receptors are capable of activating HBV biosynthesis in non-hepatoma cells

A HBV DNA (4.1 kbp) construct that can encode the four HBV transcripts and supports viral replication in hepatoma cells and hepatocytes of transgenic mice were examined for its ability to support viral transcription and replication in the human embryonic kidney 293T cell line (Fig 4) (29, 67, 89). In the absence of nuclear receptor expression vectors, the HBV 3.5 kb pregenomic RNA is not expressed and viral replication is not apparent in transient transfection analysis (Fig 4B and C, lane 1). HBV RNAs larger than 3.5 kb of unknown origin are apparent and may represent greater-than-genome length transcripts originating from the X-gene promoter (Fig 4B) (18). The HBV 2.1 kb 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 (73, 76, 89). In contrast, HNF4α, RXRα/PPARα, RXRα/FXRα, LRH1, ERRβ and ERRγ, stimulate transcription of the HBV 3.5 kb RNA and this is associated with the synthesis of encapsidated viral replication intermediates (Fig 4B and C). ERRα failed to stimulate transcription of the HBV 3.5 kb RNA and did not support viral replication in the human embryonic kidney 293T cell line (Fig 4B 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.
Figure 4. **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 1062-3182/1-1990 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. EnhL/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 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, clofibric acid and chenodeoxycholic acid at 1mM, 1mM, and 100mM, respectively, were used to activate the nuclear receptors, RXRα, PPARα and FXRα. Cells were transiently transfected with the HBV DNA (4.1kbp) construct and the indicated nuclear receptor expression vectors.
3.1.2 RXRα/FXRα activation of HBV biosynthesis in non-hepatoma cells through the DR1

To investigate further the mechanism of modulation of viral replication by the different nuclear receptors, a HBV DNA (4.1 kbp) construct (Fig 4A) 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 5A) (89). The mutation blocks nuclear receptor binding to the nucleocapsid promoter proximal HNF4 binding site and inhibits HNF4- and RXRα/PPARα-mediated HBV 3.5 kb pregenomic RNA synthesis and viral replication (Fig 5, lanes 3–6) (89). The nuclear hormone receptor-dependent level of HBV 3.5 kb 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, RXRα/PPARα and RXRα/FXRα (Fig 5A, lanes 3–8) but changed only to a very limited extent with the expression of LRH1, ERRβ and ERRγ (Fig 5A, lanes 9 and 10, and 13–16). As observed in mouse fibroblasts, HNF4α- and RXRα/PPARα-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 5B, lanes 3–6) (89). RXRα/FXRα-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 HNF4α and RXRα/PPARα (Fig 5B, lanes 3–8). In contrast, LRH1-, ERRβ- and ERRγ-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 5B, lanes 9 and 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, HNF4α, RXRα/PPARα and RXRα/FXRα, but is considerably less important for viral transcription and replication mediated by the monomeric nuclear receptors, LRH1, ERRβ and ERRγ. 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 3.5 kb 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 3.5 kb pregenomic RNA compared with the HBV 3.5 kb precore RNA (89).
Figure 5. 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, HNF4α; lane 5-6, RXRα plus PPARα; lanes 7-8, RXRα plus FXRα; lanes 9-10, LRH1; lanes 11-12, ERRα; lanes 13-14, ERRβ; lanes 15-16, ERRγ. 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 (89). 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.
Two RXRα/FXRα binding sites have been identified within the nucleocapsid promoter (Fig 2) (71). Therefore it appears that binding to the proximal RXRα/FXRα recognition site, which overlaps with the proximal HNF4α recognition site, contributes the majority of the transcriptional activity whereas binding to the distal site contributes the remaining RXRα/FXRα-mediated promoter activity. These observations indicate that the dimeric nuclear receptors, HNF4α, RXRα/PPARα and RXRα/FXRα, 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 5).

3.1.3 SHP inhibition and PGC1α activation of RXRα/FXRα activated HBV biosynthesis in non-hepatoma cells

Transfection of the HBV DNA (4.1kbp) construct with the RXRα and FXRα expression vectors into 293T cells supports HBV transcription and replication (Fig 6A and C, lane 1). Expression of increasing levels of PGC1α activates whereas SHP inhibits both HBV 3.5-kb RNA synthesis and viral replication in a dose-dependent manner (Fig 6). Increasing the levels of PGC1α enhanced viral biosynthesis at all levels of SHP expression in a similar manner (Fig 6). However, SHP inhibition was relatively ineffective at the lower levels examined and was only readily apparent at the highest level of SHP expression (Fig 6) suggesting that RXRα/FXRα-mediated viral replication in 293T cells is relatively insensitive to SHP mediated inhibition.
Figure 6. Effect of PGC1α and SHP expression of HBV biosynthesis in the human embryonic kidney cell line, 293T, expressing RXRα/FXRα. Cells were transfected with the HBV DNA (4.1kbp) construct plus the RXRα and FXRα expression vectors (lane 1) or the HBV DNA (4.1kbp) construct plus the RXRα, FXRα, PGC1α and SHP expression vectors (lanes 2-16) 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) Quantitative analysis of the HBV 3.5-kb RNA from three independent experiments. Trend lines were calculated using linear regression analysis. (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 three independent experiments. Trend lines were calculated using linear regression analysis. All-trans retinoic acid and chenodeoxycholic acid at 1mM and 100mM, respectively, were used to activate the nuclear receptors, RXRα and FXRα.
3.2 **HBV biosynthesis in SHP-expressing and SHP-null HBV transgenic mice**

3.2.1 **Effect of bile acid feeding on FXR and SHP regulated gene expression in SHP-expressing and SHP-null HBV transgenic mice**

HBV transgenic mice were bred with SHP-null mice and HBV transgenic mice hemizygous for the HBV transgene and heterozygous (+/-) for the wild-type SHP-expressing allele or homozygous (-/-) for the SHP-null allele were identified in the F2 generation. In these studies, both SHP-expressing and SHP-null HBV transgenic mice were fed a diet supplemented with 1% (wt/wt) cholic acid for 7 days (94). Male and female mice of each genotype were assayed for the levels of liver SHP, FXRα, BSEP, CYP7A1 and PGC1α RNAs after bile acid feeding and compared with animals fed a control diet (Fig 7). As expected, mice heterozygous for the SHP-expressing allele displayed an approximately two-fold induction of SHP RNA after bile acid feeding (Fig 7) (94). SHP-null HBV transgenic mice did not express SHP RNA (Fig 7A). In both male and female HBV transgenic mice, the abundance of the FXRα RNA appeared to be very modestly increased due to the absence of SHP (Fig 7B). Importantly, the inclusion of cholic acid in the diet was associated with a decrease in FXRα transcript levels as previously noted (94). Additionally, cholic acid appeared to induce the expression of the bile salt export pump (BSEP) transcript in SHP-expressing HBV transgenic mice and this induction was not apparent in the SHP-null HBV transgenic mice (Fig 7C) (94). Most notably, feeding mice a diet which
included cholic acid resulted in a dramatic inhibition of CYP7A1 expression that was relieved to a limited extent in the SHP-null mice (Fig 7D) (94). These observations clearly indicate that the cholic acid diet was effectively modulating FXR and SHP activities in the liver in an attempt to maintain normal bile acid homeostasis as previously noted (Fig 3) (56, 94).

As PGC1α is a coactivator that can counteract the effect of SHP on HBV biosynthesis in cell culture (Fig 5) and its expression has previously been shown to be down regulated in vivo by bile acids (57). The effect of feeding cholic acid on this transcript was examined in the HBV transgenic mice (Fig 7E). Contrary to previous observations, cholic acid treatment did not display any consistent effects on PGC1α RNA levels in these mice suggesting this coactivator probably does not play a role in modulating the level of HBV biosynthesis under these particular physiological circumstances. The reason PGC1α RNA levels were unaffected in the HBV transgenic mice is unclear but may reflect differences in the genetic backgrounds of the mice used in the various studies or the methods used to measure the levels of the PGC1α transcript.
Figure 7. Analysis of SHP, FXRα, BSEP, CYP7A1 and PGC1α transcripts in the livers of HBV transgenic mice. Mice were fed a control (Control) or 1% (wt/wt) cholic acid (CA) diet for 7 days. Quantitative analysis of the (A) SHP, (B) FXRα, (C) BSEP, (D) CYP7A1 and (E) PGC1α transcripts by RT-qPCR in the HBV transgenic mice. The GAPDH transcript was used as an internal control for the quantitation of the SHP, FXRα, BSEP, CYP7A1 and PGC1α RNAs. The mean relative SHP, FXRα, BSEP, CYP7A1 and PGC1α transcript levels plus standard deviations derived from male and female SHP-expressing (+/-) and SHP-null (-/-) HBV transgenic mice are shown. The levels of the transcripts in the cholic acid fed HBV transgenic mice which are statistically significantly different from their levels in the corresponding SHP-expressing or SHP-null HBV transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
3.2.2 Effect of bile acid feeding on serum HBeAg, liver damage and inflammation in HBV transgenic mice

HBV transgenic mice displayed a statistically significant increase of approximately two-fold in the level of serum HBeAg after bile acid feeding as compared to the level of serum HBeAg immediately prior to the addition of cholic acid to the control diet (Fig. 8A). All 22 individual mice examined demonstrated an increase in serum HBeAg as a consequence of bile acid feeding varying from a 10% to 300% elevation in serum HBeAg level. The increase in serum HBeAg appeared to be more pronounced in the SHP-null HBV transgenic mice than the SHP-expressing HBV transgenic mice although the levels of serum HBeAg in the bile acid treated SHP-expressing and SHP-null HBV transgenic mice were only statistically significant in the male mice (Fig. 8A). As HBeAg is translated from the HBV 3.5kb precore RNA (63), these observations suggest that the diet supplemented with cholic acid may have a modest effect on the synthesis of the HBV 3.5kb precore RNA in the HBV transgenic mice.
Figure 8. Effect of bile acid feeding on serum HBeAg, ALT levels and TNFα transcripts in the livers of HBV transgenic mice. (A) Serum HBeAg levels were measured on day 0 (d0) and day 7 (d7) of the 1% cholic acid diet. The mean HBeAg levels plus standard deviations derived from male and female SHP-expressing (+/-) and SHP-null (-/-) HBV transgenic mice are shown. The levels of serum HBeAg in the cholic acid fed HBV transgenic mice from day 0 to day 7 which are statistically significantly different by a paired Student’s t-test (p<0.05) are indicated with an asterisk (*). (B) Serum ALT levels were measured on day 0 (d0), day 4 (d4), day 6 (d6) and day 7 (d7) of the 1% cholic acid diet (Units per liter, U/L). The mean ALT levels plus standard deviations derived from the initial pretreatment (d0) and peak activity sera samples (d4-6) from male and female SHP-expressing (+/-) and SHP-null (-/-) HBV transgenic mice are shown. (C) Quantitative analysis of the TNFα transcripts by RT-qPCR in the HBV transgenic mice. The GAPDH transcript was used as an internal control for the quantitation of the TNFα RNA. The mean relative TNFα transcript levels plus standard deviations derived from male and female SHP-expressing (+/-) and SHP-null (-/-) HBV transgenic mice are shown.
As bile acid feeding can be associated with hepatic cytotoxicity (95), the levels of serum alanine aminotransaminase (ALT) were examined (Fig 8B). Indeed, the bile acid fed mice displayed a marked increase in serum ALT, which appeared more pronounced in the male SHP-null HBV transgenic mice. The peak serum ALT level was observed between days 4 and 6 and declined up to two-fold by day 7 suggesting there was a protective homeostatic adjustment to increased bile acid intake occurring in the liver (56, 94). The observed elevation in serum ALT raised the possibility that the increase in serum HBeAg observed in the cholic acid treated HBV transgenic mice might be due to the destruction of hepatocytes replicating virus, although histological examination of these livers appeared normal. Indeed, chemically-induced liver damage in HBV transgenic mice is associated with an increase in both serum ALT and HBeAg levels (Reese and McLachlan, data not shown). In addition, it was possible that hepatic cell death might be associated with increased inflammation although leukocyte infiltrates were not observed in these livers. To examine the possibility that hepatic Kupffer cells were becoming activated as a result of hepatic damage, the level of TNFα RNA in the livers of these mice was measured (Fig 8C). No significant elevation in TNFα gene expression was observed indicating the absence of any inflammatory response in the liver in response to the cholic acid diet.
3.2.3 **Effect of bile acid feeding on viral transcription in HBV transgenic mice**

HBV transgenic mice that were heterozygous or homozygous for the SHP-null allele were examined for their steady state levels of HBV transcripts by analysis of the total liver RNA (Fig 9). The steady state levels of the HBV 3.5- and 2.1kb transcripts in the livers of the HBV transgenic mice with and without SHP were not greatly influenced by the cholic acid diet (Fig 9). Measurement of the levels of the HBV 3.5kb transcripts indicated they were increased about 1.9-fold by the cholic acid diet in male SHP-expressing HBV transgenic mice based on the RNA filter hybridization (Fig. 9B) and RT-qPCR analysis (Fig 9C). These observations are consistent with the suggestion that bile acids activate FXR leading to increase transcription from the HBV nucleocapsid promoter (Fig 3-5) (71). In the male SHP-null HBV transgenic mice, the levels of the HBV 3.5kb transcripts were 2.8- and 1.3-fold higher than those observed in the SHP-expressing male HBV transgenic mice based on the RNA filter hybridization (Fig 8B) and RT-qPCR analysis (Fig 9C). These observations are consistent with the suggestion that under normal physiological conditions SHP can modestly suppress nuclear receptor-mediated transcription from the HBV nucleocapsid promoter (Fig 3,6) (67). Male SHP-null HBV transgenic mice fed the cholic acid diet did not display a greater increase in the HBV 3.5kb transcripts than the SHP-expressing HBV transgenic mice fed the cholic acid diet or the SHP-null HBV transgenic mice fed the control diet suggesting that the activation of FXR by bile acids in the absence of the nuclear receptor corepressor, SHP, did not lead to an
enhanced effect on HBV transcription as might have been predicted (Fig 3). In female HBV transgenic mice, it was interesting to note that neither the cholic acid diet nor the absence of SHP increased the level of the HBV 3.5kb transcripts suggesting this transcriptional response to bile acids may be sexually dimorphic in nature in the liver (Fig 9).
A

HBV 3.5kb RNA
HBV 2.1kb RNA
GAPDH RNA

Sex: Male Female
SHP genotype: +/- +/- +/- +/-
Cholic acid: - + - + - + - +

B

![Bar graph showing HBV 3.5kb RNA/GAPDH ratio in male and female HBV transgenic mice.](image)

C

![Bar graph showing HBV 3.5kb RNA/GAPDH ratio (RT-qPCR) in male and female HBV transgenic mice.](image)
Figure 9. RNA (Northern) filter hybridization and RT-qPCR analysis of HBV transcripts in the livers of HBV transgenic mice. (A) RNA (Northern) filter hybridization of groups of two representative mice of each sex and genotype are shown. The probes used were HBVayw genomic DNA plus GAPDH cDNA. SHP-expressing (+/-) HBV transgenic mice heterozygous for the SHP allele and SHP-null (-/-) HBV transgenic mice are indicated. Mice were fed a control (-) or a 1% (wt/wt) cholic acid (+) diet for 7 days. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for the quantitation of the HBV 3.5kb RNA. (B) Quantitative analysis of the HBV 3.5kb transcript in the HBV transgenic mice. The mean HBV 3.5kb transcript levels plus standard deviations derived from 6 control male SHP (+/-) HBV transgenic mice, 7 cholic acid fed male SHP (+/-) HBV transgenic mice, 8 control male SHP (-/-) HBV transgenic mice, 4 cholic acid fed male SHP (-/-) HBV transgenic mice, 3 control female SHP (+/-) HBV transgenic mice, 6 cholic acid fed female SHP (+/-) HBV transgenic mice, 5 control female SHP (-/-) HBV transgenic mice and 5 cholic acid fed female SHP (-/-) HBV transgenic mice are shown. The levels of the HBV 3.5kb transcript in the cholic acid fed HBV transgenic mice which are statistically significantly different from their levels in the corresponding SHP-expressing or SHP-null HBV transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*). The level of the HBV 3.5kb RNA detected in the control male SHP (+/-) HBV transgenic mice were also statistically significantly different from the levels in the control male SHP (-/-) HBV transgenic mice by the Student’s t-test (p<0.05). (C) Quantitative analysis of the HBV 3.5kb transcript by RT-qPCR in the HBV transgenic mice analyzed in (B). The GAPDH transcript was used as an internal control for the quantitation of the HBV 3.5kb RNAs. The mean relative HBV 3.5kb transcript levels plus standard deviations are shown. The levels of the transcripts in the cholic acid fed HBV transgenic mice which are statistically significantly different from their levels in the corresponding SHP-expressing (+/-) or SHP-null (-/-) HBV transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
3.2.4 Effect of bile acid feeding on viral replication intermediates in HBV transgenic mice

The alterations in the levels of replication intermediates in the livers of SHP-expressing and SHP-null HBV transgenic mice on control or cholic acid diets closely paralleled the observed changes in HBV 3.5kb RNA abundances (Figs 9 and 10). Measurement of the levels of the HBV replication intermediates indicated they were increased about 1.3-fold by the cholic acid diet in male SHP-expressing HBV transgenic mice based on the DNA filter hybridization analysis (Fig 10B). In the male SHP-null HBV transgenic mice, the levels of the HBV replication intermediates were 2.3-fold higher than those observed in the SHP-expressing male HBV transgenic mice based on the DNA filter hybridization analysis (Fig 10B).

These observations are consistent with the transcriptional analysis (Fig 9) and suggest that cholic acid may activate and SHP may inhibit HBV 3.5kb RNA synthesis from the HBV nucleocapsid promoter to modulate HBV biosynthesis (Fig 3,6) (67). As observed with the HBV 3.5kb RNA synthesis, female HBV transgenic mice displayed very limited, statistically insignificant, alterations in viral replication intermediates in response to the cholic acid diet or the loss of SHP (Fig 10). These observations suggest that the transcriptional regulatory network involving FXR and SHP, which helps to maintain bile acid homeostasis within the liver, may preferentially modulate HBV transcription and replication in males, possibly contributing to the greater disease burden in men relative to women associated with chronic HBV infection (26, 43, 55, 56, 100).
A

HBV Tg DNA
HBV RC DNA
HBV SS DNA

Sex:

Male
Female

SHP genotype:

+/-
-/
+/-
-/

Cholic acid:

-
+
-
+

B

Replication intermediates (RI/Tg)

[Bar graph showing replication intermediates for male and female HBV transgenic mice with different SHP genotypes.]

[Legend:

- SHP(+/-) Control
- SHP(+/-) CA
- SHP(-/-) Control
- SHP(-/-) CA]
Figure 10. **DNA (Southern) filter hybridization analysis of HBV DNA replication intermediates in the livers of HBV transgenic mice.** (A) DNA (Southern) filter hybridization of groups of two representative mice of each sex and genotype are shown. The probe used was HBV ayw genomic DNA. SHP-expressing (+/-) HBV transgenic mice heterozygous for the SHP allele and SHP-null (-/-) HBV transgenic mice are indicated. Mice were fed a control (-) or a 1% (wt/wt) CA (+) diet for 7 days. The HBV transgene (Tg) was used as an internal control for the quantitation of the HBV replication intermediates. Tg = HBV transgene; RC = HBV relaxed circular replication intermediates; SS = HBV single stranded replication intermediates. (B) Quantitative analysis of the HBV DNA replication intermediate (RI) levels in HBV transgenic mice. The mean HBV DNA replication intermediate levels plus standard deviations derived from 6 control male SHP (+/-) HBV transgenic mice, 7 CA fed male SHP (+/-) HBV transgenic mice, 8 control male SHP (-/-) HBV transgenic mice, 4 CA fed male SHP (-/-) HBV transgenic mice, 3 control female SHP (+/-) HBV transgenic mice, 6 CA fed female SHP (+/-) HBV transgenic mice, 5 control female SHP (-/-) HBV transgenic mice and 5 CA fed female SHP (-/-) HBV transgenic mice are shown. The levels of the replication intermediates in the CA fed HBV transgenic mice were not statistically significantly different from their levels in the corresponding SHP-expressing (+/-) or SHP-null (-/-) HBV transgenic mice by a Student’s t-test (p<0.05). The level of replication intermediates detected in the control male SHP (+/-) HBV transgenic mice were statistically significantly different from the levels in the control male SHP (-/-) HBV transgenic mice by the Student’s t-test (p<0.05).
3.3 **Absence of synergistic modulation of HNF4 and FXR directed HBV biosynthesis in human embryonic kidney 293T cells**

Bile acid feeding in the presence or absence of SHP has a modest effect on viral transcription and replication in male HBV transgenic mice and essentially no effect on viral biosynthesis in female HBV transgenic mice (Figs 9 and 10). This appears contrary to the effects observed in cell culture where FXR and SHP can greatly modulate HBV biosynthesis (Fig 6) (67). However to more closely approximate the conditions observed *in vivo*, the consequence of expressing multiple nuclear receptors on HBV biosynthesis was investigated in nonhepatoma cells (Fig 11). Transfection of the replication competent HBV DNA (4.1kbp) construct alone into 293T cells untreated or treated with bile acid failed to support viral biosynthesis (Fig 11, lane 1 and 2). Transfection of the HBV DNA (4.1kbp) construct with an HNF4 expression vector in the absence, or presence, of bile acid supported HBV transcription and replication (Fig 11, lane 3 and 4). Transfection of the HBV DNA (4.1kbp) construct with a RXRα plus FXRα expression vector in the presence, but not in the absence, of bile acid supported robust HBV transcription and replication (Fig 11, lane 5 and 6). Critically, transfection of the HBV DNA (4.1kbp) construct with an HNF4α, RXRα and FXRα expression vectors in the presence of bile acid supported a level of HBV transcription and replication that was similar to that observed with HNF4α or RXRα plus FXRα expression vectors alone (Fig 11, lane 8). This indicates that HNF4α and RXRα plus FXRα do not synergistically or even additively cooperate to promote HBV transcription.
Figure 11. **HNF4 and FXRα directed HBV biosynthesis in human embryonic kidney 293T cells.** (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. Chenodeoxycholic acid (CDCA) at 100mM was used to activate the nuclear receptor, FXRα. Cells were transiently transfected with the HBV DNA (4.1kbp) construct and the indicated nuclear receptor expression vectors. Lane 1, Control; lane 2, Control plus CDCA; lane 3, HNF4α; lane 4, HNF4α plus CDCA; lane 5, RXRα/FXRα; lane 6, RXRα/FXRα plus CDCA; lane 6, RXRα/FXRα plus HNF4α; lane 8, RXRα/FXRα plus CDCA plus HNF4α.
This observation may explain the limited effect of bile acid treatment on HBV biosynthesis in the HBV transgenic mice. HNF4α, and other nuclear receptors such as LRH1 and ERR, which are capable of supporting HBV biosynthesis are constitutively expressed in the liver. Activation of FXR by bile acids must compete with these additional nuclear receptors to activate transcription from the HBV nucleocapsid promoter (Fig 3). Based on the cell culture analysis (Fig 11), it is possible that *in vivo* even the activation of FXR by bile acids is not sufficient to further enhance HBV transcription and replication which is being directed by the constitutively active nuclear receptors such as HNF4 that appear to be essential for viral biosynthesis (Fig 3) (51). Interestingly the expression of HNF4 and RXRα plus FXRα in the absence of bile acid appears to support less HBV RNA and DNA synthesis than observed with HNF4 alone (Fig 11, lanes 3, 5 and 7) suggesting that RXRα plus FXRα in the absence of ligand may actually inhibit HNF4-mediated viral biosynthesis, presumably by competing for the proximal nuclear receptor binding site in the HBV nucleocapsid promoter.
4. ROLE OF FOXA IN HBV BIOSYNTHESIS

4.1 Down regulation of FoxA in HNF4α-deficient mice

One reason to assess the role of FoxA in HBV biosynthesis (see Section 1.3.5) is the recent finding that FoxA can support HBV biosynthesis in a non-hepatoma HBV replication system when the specific coactivator, PGC1α, is cotransfected with the FoxA expression vector (Ondracek and McLachlan, personal communication). Furthermore, HBV transgenic mice with a liver-specific conditional knockout of HNF4α do not exhibit any HBV transcription and replication demonstrating that HNF4α is essential for viral biosynthesis in vivo. Although HNF4α is essential for viral biosynthesis in early neonatal development in the HBV transgenic mouse, it is still not established if the effects of HNF4α in vivo are direct or indirect. However, it is apparent that the effect of HNF4α occurs at the level of viral transcription as the loss of this nuclear receptor is associated with a corresponding decrease in HBV transcription (50). If the effect of HNF4α is not directly on viral transcription, it most likely affects this process by altering the activities of additional factors capable of supporting HBV biosynthesis. The loss of HNF4α has been previously reported to lead to decreased FoxA2 expression (32, 70). Therefore, a reduction in FoxA expression is a potential indirect mechanism by which loss of HNF4α in development leads to an absence of HBV biosynthesis in the HBV transgenic mouse. Thus, the mRNA levels of FoxA1, FoxA2 and FoxA3 in the HNF4α-deficient HBV transgenic mice were measured by RT-qPCR. It was shown that decreased mRNA levels of HNF4α correlated with decreased mRNA levels in all three of the FoxA
factors (Fig 12). This finding further advocated the necessity to determine the role of FoxA in HBV biosynthesis in vivo using a FoxA-deficient HBV transgenic mouse model.
Figure 12. Effect of liver-specific conditional deletion of HNF4α in HBV transgenic mice on FoxA levels: Effect of HNF4α RNA levels in the liver on FoxA levels in the liver both measured by reverse transcription-quantitative polymerase chain reaction. Cre(-), control HBVAlbCre(-)HNF4α(fl/fl) mice; Cre(+), HBVAlbCre(+)HNF4α(fl/fl) mice with approximately wild-type phenotype; sCre(+), HBVAlbCre(+)HNF4α(fl/fl) mice with the small phenotype. (A) HNF4α levels compared to FoxA1 levels (B) HNF4α levels compared to FoxA2 levels (C) HNF4α levels compared to FoxA3 levels.
4.2 **Breeding of FoxA HBV transgenic mice**

Floxed FoxA1, floxed FoxA2 and FoxA3-null mice were a generous gift from Dr. Klaus H. Kaestner, University of Pennsylvania School of Medicine (37, 86). Several strains of FoxA-deficient HBV transgenic mice have been developed. These include (i) liver-specific FoxA1-null, (ii) liver-specific FoxA2-null, (iii) FoxA3-null, (iv) liver-specific FoxA1+FoxA2-null and (v) liver-specific FoxA1+FoxA2-null plus FoxA3(+-) HBV transgenic mice. Liver-specific loss of FoxA1, FoxA2 and FoxA3 failed to generate viable mice for this study. Deletion of FoxA1 or FoxA3 has no effect on HBV biosynthesis (Reese and McLachlan, data not shown) (50). Therefore, a focus of this study is assessing HBV biosynthesis in (i) liver-specific FoxA2-null, (ii) liver-specific FoxA1+FoxA2-null and (iii) liver-specific FoxA1+FoxA2-null plus FoxA3(+-) HBV transgenic mice. Thus, to determine the role of FoxA2 in HBV biosynthesis, we bred HBV transgenic mice with mice carrying the floxed FoxA2 gene and albumin Cre recombinase transgene (AlbCre) to generate HBV(+-)FoxA2(fl/fl)AlbCre transgenic mice. We also bred HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)AlbCre and HBV(+-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-)AlbCre transgenic mice to determine the effect of deleting both FoxA1 and FoxA2 on HBV biosynthesis and deleting all FoxA but one allele of FoxA3 on HBV biosynthesis.
4.3  **Effect of FoxA deletion on HBV biosynthesis *in vivo***

4.3.1  **Effect of FoxA deletion on HBV transcription**

Male and female mice of each genotype were assayed for the levels of HBV e antigen (HBeAg) in their sera at the time of sacrifice. This analysis demonstrated that secreted HBeAg encoded by the precore 3.5-kb transcript is decreased in all groups in both males and females (Fig 13) relative to wild type HBV transgenic mice. The only statistically insignificant decrease in HBeAg was in the male HBV(+/−)FoxA2(fl/fl) AlbCre transgenic mice. HBeAg was decreased approximately two-fold in the female HBV(+/−)FoxA2(fl/fl) AlbCre(+/−) transgenic mice compared to cre negative HBV(+/−)FoxA2(fl/fl) transgenic mice (p<.001). The HBeAg was significantly decreased approximately two-fold in HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)AlbCre(+/−) transgenic mice compared to cre negative HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl) transgenic mice in both males and females (p<.001). The greatest HBeAg decrease was seen in the HBV(+/−) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) transgenic mice. The HBeAg was significantly decreased greater than five-fold in both male and female HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)AlbCre(+/−) transgenic mice compared to cre negative HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−) transgenic mice (p<.001).
Figure 13. **Graph of HBeAg in the sera of FoxA-deficient HBV transgenic mice at sacrifice.** Groups of four or more mice of each genotype were analyzed. A2 signifies HBV(+/−)A2(fl/fl) mice, A1/A2 signifies HBV(+/−)FoxA1(fl/fl) FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/−). AlbCre- and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significant differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
FoxA-deficient mice were examined for their steady-state levels of HBV transcripts by analysis of the total liver RNA. The steady-state levels of the HBV 3.5 kb-transcript, measured by filter hybridization analysis, was decreased in all cre positive mice compared to their cre negative counterpart (Fig 14A and B). The only statistically insignificant decrease in HBV 3.5 kb-transcript was in the female HBV(+/-)FoxA2(fl/fl)AlbCre transgenic mice. The HBV 3.5 kb-transcript was decreased approximately 30% in the male HBV(+/-)FoxA2(fl/fl)AlbCre(+/-) transgenic mice compared to cre negative HBV(+/-)FoxA2(fl/fl) transgenic mice (p<.01). The HBV 3.5 kb-transcript was decreased approximately two-fold in HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) AlbCre(+/-) transgenic mice compared to cre negative HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) transgenic mice in both males and females (p<.01). The greatest HBV 3.5 kb-transcript decrease was seen in the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-) transgenic mice. The HBV 3.5 kb-transcript was significantly decreased greater than four-fold in both male and female HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-) FoxA3(+/-) transgenic mice (p<.001). This observation is consistent with the similar decrease in serum HBeAg. Furthermore, we also found this same decrease in viral RNA synthesis by RT-qPCR (Fig 14C).
Figure 14. RNA (Northern) filter hybridization analysis of HBV transcripts (panel A), graph of RNA (Northern) filter hybridization analysis of HBV transcripts (panel B), and graph of RT-qPCR of HBV 3.5kb-transcript (panel C) in the livers of FoxA-deficient HBV transgenic mice. Groups of four or more mice of each genotype were analyzed. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for the quantification of the HBV 3.5kb RNA. A2 signifies HBV(+/-)A2(fl/fl) mice, A1/A2 signifies HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-). AlbCre- and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
4.3.2 **Effect of FoxA deletion on HBV replication**

The levels of HBV replication intermediates were measured in the livers of FoxA-deficient HBV transgenic mice by southern analysis (Fig 15). This indicated that deletion of FoxA greatly affects the level of viral replication in HBV transgenic mice. The HBV replication intermediates were decreased greater than three-fold in the male and female HBV(+/-)FoxA2(fl/fl)AlbCre(+/-) transgenic mice compared to cre negative HBV(+/-)FoxA2(fl/fl) transgenic mice (p<.05). The HBV replication intermediates were significantly decreased approximately four-fold in HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) AlbCre(+/-) transgenic mice compared to cre negative HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) transgenic mice in both males and females (p<.01). The greatest decrease in HBV replication intermediates was seen in the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/-)AlbCre(+/-) transgenic mice. The HBV replication intermediates were undetectable in both the male and female HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/-)AlbCre(+/-) transgenic mice. These observations demonstrate the importance of FoxA for HBV transcription and replication.
Figure 15. DNA (Southern) filter hybridization analysis of replication intermediates (panel A) and graph of DNA (Southern) filter hybridization analysis of replication intermediates (panel B) in the livers of FoxA-deficient HBV transgenic mice. Groups of four or more mice of each genotype were analyzed. The HBV transgene (HBV Tg DNA) was used as an internal control for the quantification of the HBV relaxed circular (HBV RC DNA) and single stranded (HBV SS DNA) DNA replication intermediates. A2 signifies HBV(+/-) A2(fl/fl) mice, A1/A2 signifies HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−). AlbCre− and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
4.4 **Effectiveness of FoxA deletion**

The residual transcription observed in a limited number of hepatocytes could presumably represent the incomplete deletion of the FoxA1 and FoxA2 alleles by the Cre recombinase. Therefore, it was critical to determine the effectiveness of the FoxA deletion in these mice. The mRNA levels of FoxA1, FoxA2 and FoxA3 were assessed by RT-qPCR (Fig 16). The deletion of both FoxA1 and FoxA2 by AlbCre was extremely effective. As expected, the mRNA level of FoxA3 is decreased approximately two-fold from the cre negative HBV(+/−)FoxA1(+/−)FoxA2(+/−) transgenic mice to the cre negative HBV(+/−)FoxA1(+/−)FoxA2(+/−) transgenic mice. In addition, the level of FoxA3 mRNA is increased approximately two-fold when another FoxA factor is deleted.
Figure 16. RT-qPCR of FoxA1, FoxA2 and FoxA3. The mRNA levels of FoxA1 (panel A), FoxA2 (panel B), and FoxA3 (panel C) in the livers of FoxA-deficient HBV transgenic mice are shown. Groups of four or more mice of each genotype were analyzed. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control. A2 signifies HBV(+/-) A2(fl/fl)mice, A1/A2 signifies HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/-). AlbCre- and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
4.5 **Effect of FoxA deletion on transcriptional regulators of HBV biosynthesis**

One potential indirect mechanism by which the loss of FoxA might decrease HBV biosynthesis is through the down regulation of transcriptional activators of HBV biosynthesis or through up regulation of transcriptional repressors of HBV biosynthesis. As indicated (see Section 1.1.4 and Figure 4), multiple transcription factor binding sites have been identified on the HBV promoters and enhancers, and some nuclear receptors have been shown to be capable of mediating HBV biosynthesis (3, 10, 52, 71, 77, 89, 90, 106). Therefore, the mRNA levels of the transcriptional regulators of HBV biosynthesis were measured comparing the different groups of FoxA-deficient HBV transgenic mice with wild type HBV transgenic mice. Utilizing RT-qPCR to examine this issue, the levels of HNF4α, FXRα, and LRH-1 transcripts were shown to be largely unaffected by alterations in the levels of FoxA expression in the various FoxA-deficient mice (Fig 16). Additionally the mRNA levels of the corepressor, SHP, and the coactivator, PGC1α, were assessed by RT-qPCR and were found to decrease and increase approximately 2-fold, respectively, in the HBV(+/−) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) compared with the same strain lacking the AlbCre transgene (p<.05) (Fig 17). While a decrease in SHP mRNA could suggest a potential down regulation in the activity of FXRα, these alterations in corepressor and coactivator mRNA levels cannot explain the loss of HBV transcription and replication in the FoxA-deficient HBV transgenic mouse.
Figure 17. RT-qPCR of transcriptional regulators of HBV biosynthesis. The mRNA levels of HNF4α (panel A), FXRα (panel B), LRH-1 (panel C), SHP (panel D), and PGC1α (panel E) in the livers of FoxA-deficient HBV transgenic mice are shown. Groups of four or more mice of each genotype were analyzed. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control. A2 signifies HBV(+/−)FoxA2(fl/fl) mice, A1/A2 signifies HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−). AlbCre− and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
4.6 **Biochemical analysis and histology of FoxA HBV transgenic mice**

Immunohistochemical analysis of the livers from the liver-specific FoxA1+FoxA2-null plus FoxA3(+/−) HBV transgenic mice supports the contention that viral biosynthesis has been lost from the vast majority of hepatocytes (Fig 18). Furthermore, trichrome staining demonstrates that these mice have extensive fibrosis (Fig 18). The same level of fibrosis was observed in liver-specific FoxA1+FoxA2-null plus FoxA3(+/−) HBV negative mice (Reese and McLachlan, data not shown), confirming that this phenotype is a result of the FoxA-deficiency and not a consequence of the presence of HBV. Despite these histological observations, hepatocyte function in the liver-specific FoxA1+FoxA2-null plus FoxA3(+/−) HBV transgenic mice appeared relatively normal. Serum analysis of AST and ALT levels indicated no apparent hepatocyte damage and biochemical profiling showed that glucose and triglyceride levels were relatively normal with no difference between cre negative and cre positive mice (data not shown). Overall these observations support the suggestion that hepatocyte metabolic function is generally unchanged under normal physiological conditions. Of note, significant lymphocyte infiltration was not obviously apparent in these livers suggesting the loss of HBV transcription and replication was either cell autonomous or due to signaling from the nonparenchymal liver cells.
Figure 18. **Immunohistochemical staining of liver from 7 week old HBV(+/−) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) mice**: Low magnification (X10). Liver images on the left and right are wild type and mutant (liver-specific loss of FoxA1 plus FoxA2 with heterozygosity in the FoxA3 gene), respectively. Liver images on the top and bottom are stained with hepatitis B core antibody (HBcAb) (to detect hepatitis B core antigen) and Trichrome stain (to display fibrosis), respectively.
4.7 **Effect of FoxA deletion on fibrosis markers and markers of biliary epithelial cell proliferation**

The observation that the HBV transgenic mice expressing a single allele of FoxA3 (HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-)) failed to support viral biosynthesis indicated that FoxA performed an essential function, either directly or indirectly, that was necessary for HBV transcription and hence replication. It may be that FoxA directly activates HBV transcription in the context of the appropriate coactivators. However, the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-) mouse displays fibrosis which is more dramatic than observed in the previously reported FoxA1/2-null mice (53). This raises the possibility that paracrine signaling molecules synthesized by the proliferating nonparanchymal cells including various cytokines and growth factors may contribute to the loss of viral biosynthesis in this FoxA-deficient HBV transgenic mouse. Indeed, we have demonstrated that the FoxA-deficient HBV transgenic mice synthesize significant amounts of fibrosis markers in the liver by RT-qPCR (Fig 19). One marker of fibrosis, $\alpha$ smooth muscle actin ($\alpha$SMA), is significantly increased approximately four-fold in the male HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-) mice compared to the cre negative HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/-) mice (p<.01), and was significantly increased approximately six-fold in the female HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-) mice compared to cre negative HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-) transgenic mice (p<.01) (Fig 19A). Another marker of fibrosis, collagen 1a1, was significantly increased in both the
HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)AlbCre(+/−) compared to the corresponding cre negative HBV transgenic mice controls and the HBV(+/−)FoxA1(fl/fl)FoxA2(+/−)AlbCre(+/−) compared to the corresponding cre negative HBV transgenic mice controls in both males and females (Fig 19B). The mRNA levels of all isoforms of TGF-β are increased in both the HBV(+/−)FoxA1(fl/fl)FoxA2(+/−)AlbCre(+/−) compared to the corresponding cre negative HBV transgenic mice controls and the HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) compared to the corresponding cre negative HBV transgenic mice controls in both males and females (Fig 19 C-E).
Figure 19. **RT-qPCR of fibrosis markers.** The mRNA levels of αSMA (panel A), Collagen 1a1 (panel B), TGF-β1 (panel C), TGF-β2 (panel D), and TGF-β2 (panel E) in the livers of FoxA-deficient HBV transgenic mice are shown. Groups of four or more mice of each genotype were analyzed. The GAPDH transcript was used as an internal control. A2 signifies HBV(+/−)A2(fl/fl) mice, A1/A2 signifies HBV(+/−)FoxA1(fl/fl) FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/−) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−). AlbCre− and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
The HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-)AlbCre(+-) transgenic mice appear to be very similar in many regards to the FoxA1/2-deficient mice except the fibrosis and associated with biliary epithelial cell (cholangiocyte) proliferation appear more extreme than described previously (53). Consequently, the levels of biliary epithelial cell proliferation were assessed by RT-qPCR (Fig 20). Cytokeratin 19 (CK19) mRNA was increased 28-fold in male HBV(+/-) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) compared to HBV(+/-) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-) transgenic mice (p=.076) (Fig 20A). In females, CK19 mRNA was increased approximately six-fold in HBV(+/-) FoxA1(fl/fl)FoxA2(fl/fl)AlbCre(+-) compared to HBV(+/-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-) transgenic mice (p<.01) and increased approximately 17-fold in the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) compared to HBV(+/-) FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-) transgenic mice (p<.05).

Another biliary epithelial cell marker, cytokeratin 20 (CK20) was also assessed by RT-qPCR. CK20 mRNA was found to be undetectable in control mice and increased to almost glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+-)AlbCre(+-) transgenic mice (Fig 20B).
Figure 20. **RT-qPCR of biliary epithelial cell markers.** The mRNA levels of CK19 (panel A), and CK20 (panel B) in the livers of FoxA-deficient HBV transgenic mice are shown. Groups of four or more mice of each genotype were analyzed. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control. A2 signifies HBV(+/−)A2(fl/fl) mice, A1/A2 signifies HBV(+/−)FoxA1(fl/fl) FoxA2(fl/fl) and A1/A2/A3 signifies HBV(+/−) FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/−). AlbCre− and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
4.8 Effect of FoxA deletion on cytokines

The effect of a variety of stimuli leading to cytokine production has been studied for their effects on HBV biosynthesis in the HBV transgenic mouse models system (11, 27, 91). In general, it has been shown that tumor necrosis factor α (TNFα), interferon α/β (IFNα/β) and interferon γ (IFNγ) lead to the almost complete loss of viral replication intermediates within the livers of HBV transgenic mice (11, 27, 62, 91). However, these cytokines fail to have a major effect on viral transcription leading to the suggestion that they inhibit HBV biosynthesis at a posttranscriptional step in the viral life cycle (96). The mRNA level of TNFα was measured by RT-qPCR and the TNFα mRNA level of a mouse treated with lipopolysaccharide (LPS) was used for comparison (Fig 21A). In males, TNFα mRNA was increased almost two-fold in HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)AlbCre(+/-) transgenic mice compared to the corresponding cre negative controls (p<.05) and increased approximately 24-fold the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/+)AlbCre(+/-) transgenic mice compared to the corresponding cre negative controls (p<.01). In females, TNFα mRNA was increased almost four-fold in HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/+)AlbCre(+/-) transgenic mice compared to the corresponding cre negative controls (p<.001) and increased approximately 13-fold the HBV(+/-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/+)AlbCre(+/-) transgenic mice compared to the corresponding cre negative controls (p<.001). Additionally, the mRNA level of 2’-5’-oligodenylate synthetase (20AS) was measured by RT-qPCR and the 20AS mRNA level of a mouse treated with polyinosinic:polycytidylic acid (polyI:C) was used for
comparison (Fig 21B). In males, 20AS mRNA was increased approximately three-fold the HBV(+/−)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) transgenic mice compared to the corresponding cre negative controls (p<.001). In females, 20AS mRNA was increased almost three-fold in HBV(+/−)FoxA1(fl/fl) FoxA2(fl/fl)AlbCre(+/−) transgenic mice compared to the corresponding cre negative controls (p<.01) and increased approximately 11-fold in HBV(+/−) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) transgenic mice compared to the corresponding cre negative controls (p<.05).

Furthermore, since the HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−) AlbCre(+/−) transgenic mice appear to be very similar in many regards to the previously described FoxA1/2-deficient mice with fibrosis and associated with biliary epithelial cell (cholangiocyte) proliferation, the levels of interleukin 6 (IL-6) were assessed by RT-qPCR, using a mouse treated with LPS as a positive control (Fig 21C) (53). The only significant increase in IL-6 mRNA was an almost three-fold increase in the female HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+/−)AlbCre(+/−) transgenic mice compared to the corresponding cre negative controls (p<.05). The other groups had small increases in IL-6 mRNA, but these increases were not significant and no group had mRNA levels of IL-6 comparable to the LPS-treated mouse.
Figure 21. **RT-qPCR of cytokines.** The mRNA levels of TNFα (panel A), 20AS (panel B), and IL-6 (panel C) in the livers of FoxA-deficient HBV transgenic mice are shown. Groups of four or more mice of each genotype were analyzed. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control. A2 signifies HBV(+/-)A2(+/fl) mice, A1/A2 signifies HBV(+/-)FoxA1(+/fl)FoxA2(+/fl) and A1/A2/A3 signifies HBV(+/-)FoxA1(+/fl) FoxA2(+/fl)FoxA3(+/-). AlbCre- and AlbCre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significantly differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
4.9 **Effect of FoxA2 adenovirus vector injection on HBV biosynthesis**

To address further the nature of the defect in the FoxA-deficient HBV transgenic mice responsible for the loss in HBV biosynthesis, an adenovirus vector expressing the FoxA2 protein was injected into FoxA-deficient HBV transgenic mice to attempt to complement their deficiency. The HBV(+-) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) mice were infected with Adenovirus-FoxA2 (88) and examined for the appearance of HBV transcription and replication. A limited number of mice were injected with Adenovirus-FoxA2 and FoxA2 levels were increased to varying extents, some mice to wild-type levels as assessed by RT-qPCR (data not shown). Although HBeAg was not significantly increased in any of these mice, HBV 3.5 kb-RNA transcript was shown to increase to a limited extent, approximately 3 fold, compared to HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+-)AlbCre(+-) mice not injected with Adenovirus-FoxA2 (data not shown). In addition, fibrosis markers, biliary epithelial cell proliferation markers and cytokine markers did not significantly change with the injection of Adenovirus-FoxA2. More mice need to be assessed to fully examine the ability of re-expression of FoxA2 in the liver to promote HBV RNA and DNA synthesis in HBV(+-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-) AlbCre(+-) transgenic mice.

4.10 **HBV biosynthesis in 2 week old FoxA-deficient HBV transgenic mice**

HBV transgenic mice expressing a single allele of FoxA3 (HBV(+/-) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-)) fail to support viral biosynthesis
and display significant fibrosis. Therefore, it is of great interest to determine if the fibrosis happens before or after the inhibition of HBV biosynthesis. Thus, mice were sacrificed at a younger age (2 weeks old) to determine what level of HBV biosynthesis is present, as well as what level of fibrosis is present. Two week old HBV(+/-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+/-)AlbCre(+/-) transgenic mice demonstrated significantly decreased HBV transcription compared to the corresponding cre negative HBV(+/-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+/-) 2 week old controls as measured by HBeAg and RT-qPCR (Fig 22 A and B). Therefore, the HBV inhibition observed as a consequence of FoxA-deficiency in HBV transgenic mice is present as early as 2 weeks. Interestingly, RT-qPCR of collagen 1a1, TGF-β1, CK19, TNFα, and 20AS revealed no significant difference between the 2 week old HBV(+/-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+/-) AlbCre(+/-) transgenic mice and the corresponding 2 week old cre negative controls (Fig 22 C-G). Only a limited number of mice have been examined at this time. However, this preliminary experiment suggests that the fibrosis per se or the cytokines and/or growth factors promoting fibrosis are not responsible for the inhibition of HBV biosynthesis observed in FoxA-deficient HBV transgenic mice. Instead this analysis supports the conclusion that the deficiency of FoxA itself plays a significant role in the inhibition of HBV biosynthesis.
Figure 22. **HBeAg and HBV RT-qPCR of 2 week old mice.** The HBeAg levels (panel A) are shown and the mRNA levels of HBV 3.5kb RNA (panel B), Collagen 1a1 (panel C), TGF-β1 (panel D), CK19 (panel E), TNFα (panel F), and 20AS (panel G) in the livers of FoxA-deficient HBV transgenic mice are shown. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control. All mice in this figure are HBV(+/-) FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/-). Cre- and Cre+ indicate the absence or presence of the liver-specific expression of the albumin Cre recombinase transgene responsible for the deletion of the floxed FoxA alleles. Statistically significant differences between cre negative and cre positive transgenic mice by a Student’s t-test (p<0.05) are indicated with an asterisk (*).
5. DISCUSSION

5.1 HBV biosynthesis in cell culture

The transcriptional regulation of HBV has been extensively studied in cell culture. As HBV transcription and replication occurs in the human hepatoma cell lines HepG2 and Huh7 when transfected with HBV DNA, it has not been readily possible to determine which transcription factors are essential for HBV biosynthesis using this approach (12, 87, 89, 93, 99). However, complementation of nonhepatoma cells with liver-enriched transcription factors has permitted the evaluation of the specific roles of individual factors in HBV transcription and replication (Fig 4-6) (89). Interesting, the HBV nucleocapsid promoter contains several recognition sites for multiple members of the nuclear receptor superfamily, including HNF4α, RXRα plus PPARα, RXRα plus FXRα, LRH1 and ERR, which have been shown to be capable of supporting HBV biosynthesis in nonhepatoma cells (Fig 4) (89). Based on results presented in this study, it is apparent that RXRα/FXRα, in addition to HNF4α and RXRα/PPARα, can direct the efficient expression of the HBV 3.5 kb 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.

The observation that multiple nuclear receptors can support transcription from the nucleocapsid promoter raised the issue of the importance of the proximal HNF4α recognition site for their activities. Two RXRα/FXRα binding sites have been identified within the nucleocapsid promoter (Fig 2) (71). Therefore it appears that binding to the proximal RXRα/FXRα recognition site, which
overlaps with the proximal HNF4α recognition site, within the nucleocapsid promoter contributes the majority of the transcriptional activity whereas binding to the distal site contributes the remaining RXRα/FXRα-mediated promoter activity (Fig 5). In addition, SHP and PGC1α modulate of RXRα/FXRα-dependent HBV biosynthesis in human embryonic kidney 293T cells (Fig 6). These findings indicate that under various physiological states e.g. fasting or choleostasis, HBV biosynthesis could be regulated by different nuclear receptors and this redundancy of function as liver physiology changes might permit constant HBV biosynthesis.

5.2 **Role of bile acids in HBV biosynthesis in vivo**

Although these cell culture studies have uncovered a previously unappreciated critical role for nuclear receptors in HBV biosynthesis, it remains unclear which members of the nuclear receptor superfamily are critical to HBV RNA and DNA synthesis in vivo. The absence of any small animal models of human HBV infection seriously limits examination of the in vivo role for nuclear receptors in viral transcription and replication. To address this problem, the HBV transgenic mouse model of chronic HBV infection was developed and shown to recapitulate many of the post-entry steps in the virus life cycle (29). Consequently, this animal model has become the most manipulatable small animal model where the in vivo role of transcription factors affecting HBV RNA and DNA synthesis can be evaluated (2, 3, 28, 50, 67, 72, 78). Indeed, the model has been used to demonstrate that HNF4α is essential for the developmental
expression of HBV biosynthesis and PPARα does not play a role in governing HBV biosynthesis under normal physiological conditions but can be activated using synthetic ligands to enhance viral transcription and replication (28, 51).

In this study (Section 3.2), the role of bile acids, their nuclear receptor FXR and the corepressor SHP in HBV biosynthesis was investigated in vivo using the HBV transgenic mouse model system. In general, activated FXR appears to have a very modest effect on HBV RNA and DNA synthesis and SHP seems to partially inhibit viral biosynthesis under normal physiological conditions in male HBV transgenic mice (Figs 9 and 10). In contrast, HBV biosynthesis is unaffected under identical conditions in the female HBV transgenic mice examined (Figs 9 and 10). This suggests there are subtle differences in the regulation of HBV biosynthesis by the bile acid-FXR-SHP regulatory network in male and female mice (Fig 3) (26, 56). The reasons for these differences are currently unclear but it is worth noting that disease progression is generally more severe in males than females indicating there are sexually dimorphic differences present both in mice and man (43, 55, 100).

In an attempt to explain the very modest effect of FXR activation on HBV biosynthesis in vivo, the relative importance of HNF4α and RXRα plus FXRα was examined in nonhepatoma cells (Fig 11). Previously, it has been shown that both these nuclear receptors can direct the expression of HBV 3.5kb RNA and hence viral replication (Figs 4-6) (89). Notably, the simultaneous expression of both these nuclear receptors does not lead to any enhancement of HBV RNA and DNA synthesis over that seen with the transcription factors alone (Fig 11). The
absence of any additive or synergistic effects on viral biosynthesis in cell culture suggests an explanation for the lack of any major effect of the bile acid diet *in vivo* (Figs 9 and 10). In the liver, HNF4 is presumably constitutively active. Activation of FXR by the bile acid diet clearly altered the expression of both FXR and SHP target genes as expected (Figs 3 and 7) (26, 56, 57). However, activated FXR had only a minimal effect on HBV biosynthesis presumably because it cannot efficiently enhance HNF4-mediate HBV nucleocapsid promoter activity (Fig 11). The ability of FXR to modestly increase viral biosynthesis in male mice but not in female mice may reflect subtle differences in the nature of the transcription factors governing HBV nucleocapsid promoter activity in the different sexes. Regardless of these differences, it is important to note that the HBV transgenic mouse model reflects only the changes in expression associated with a single viral replication cycle and even a small difference in replication efficiency can lead to a dramatic amplification in viral titer over an infection period that represents many replication cycles. Therefore it is possible that bile acid metabolism in the liver might contribute to some of the difference in viral synthesis and disease outcomes associated with HBV infections in men and women (43, 55, 100).

### 5.3 **Role of FoxA in HBV biosynthesis *in vivo***

As noted (Section 1.3.5), it has been recently demonstrated that FoxA can support HBV biosynthesis in a non-hepatoma HBV replication system when the specific coactivator, PGC1α, is cotransfected with the FoxA expression vector
Moreover, it was demonstrated (Section 4.1) that decreased mRNA levels of FoxA1, FoxA2 and FoxA3 occur in the liver-specific HNF4α-deficient HBV transgenic mice by RT-qPCR (Fig 12) (51). Therefore, since the liver-specific HNF4α-deficient HBV transgenic mice do not support HBV biosynthesis, it was of great interest to determine the role of FoxA in HBV biosynthesis in vivo. Current observations (Section 4.3) show decreased HBV biosynthesis in FoxA-deficient HBV transgenic mice (Figs 13-15). It is apparent that the liver-enriched transcription factor network that generates the hepatocyte-specific phenotype is developmentally regulated and requires both auto- and cross-regulation of the enhancer and promoter activities regulating these liver-enriched transcription factors to establish and maintain normal liver physiology (42). Disruption of the liver-enriched transcription factor network by the selective deletion of specific liver-enriched transcription factors may have limited or dramatic effects on liver physiology depending on the relative importance of the factor to the network and the additional hepatocyte-specific genes that it regulates, either directly or indirectly (42). Our initial characterization of several liver-enriched nuclear receptors including HNF4α, FXRα and LRH-1 by RT-qPCR indicated that these transcription factors are not modulated to any major extent in the FoxA-deficient HBV transgenic mice lacking HBV biosynthesis (Fig 16). Additionally the mRNA levels of the corepressor, SHP, and the coactivator, PGC1α, were assessed by RT-qPCR and were found to decrease and increase approximately 2-fold, respectively, in the HBV(+/−)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−)
transgenic mice compared with the same strain lacking the AlbCre transgene (Fig 17). While a decrease in SHP mRNA could suggest a potential small down regulation in the activity of FXRα, these alterations in corepressor and coactivator mRNA levels cannot explain the loss of HBV transcription and replication in the FoxA-deficient HBV transgenic mouse. Thus, the only transcriptional regulator of HBV biosynthesis found to change significantly in the FoxA-deficient HBV transgenic mice is FoxA.

If the loss of HBV biosynthesis in the FoxA-deficient HBV transgenic mice is the direct consequence of the reduction in the level of FoxA transcription factors, stimulation of viral biosynthesis by peroxisome proliferators or bile acids should be limited to the few cells expressing sufficient FoxA protein to support HBV transcription. Preliminary analysis of FoxA-deficient HBV transgenic mice treated with peroxisome proliferators do support the suggestion that the loss of FoxA rather than alterations in nuclear receptor activity is the primary determinant of HBV biosynthesis in this model system (Reese and McLachlan, data not shown). Also, a preliminary study demonstrated re-expression of FoxA2 using Adenovirus-FoxA2 gives a limited increase in HBV biosynthesis (section 4.9). Moreover, initial characterization of younger mice, 2 weeks old, show HBV inhibition in HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+−)AlbCre(+−) transgenic mice compared to the corresponding cre negative controls without any significant difference in fibrosis markers, biliary epithelial cell proliferation markers or cytokine markers (section 4.10). These findings further support the idea that FoxA itself is critical for HBV biosynthesis.
Immunohistochemical analysis (Section 4.6) of the livers from the liver-specific FoxA1+FoxA2-null plus FoxA3(+-) HBV transgenic mice supports the contention that viral biosynthesis has been lost from the vast majority of hepatocytes, and trichrome staining demonstrated that these mice have extensive fibrosis (Fig 18). The fibrosis observed in the HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) transgenic mice is due to stellate cell activation demonstrated by the RT-qPCR results (Fig 19). In addition, RT-qPCR results (Section 4.7) demonstrated increased markers of biliary epithelial cell proliferation (Fig 20). This raises the possibility that paracrine signaling molecules synthesized by the proliferating nonparanchymal cells including various cytokines and growth factors may contribute to the loss of viral biosynthesis in this FoxA-deficient HBV transgenic mouse.

Injection of carbon tetrachloride (CCl$_4$) induces liver injury and fibrosis in mice (92). Therefore, fibrosis was induced in wild type HBV transgenic mice using the CCl$_4$ model of fibrosis. In this model, hepatocyte necrosis occurs and TNFα is produced (Reese and McLachlan, data not shown) (64). After 6 weeks of CCl$_4$ administration, the levels of cytokines, stellate cell activation and biliary epithelial cell proliferation were evaluated by RT-qPCR analysis and were intermediate between the FoxA1 plus FoxA2 deficient HBV transgenic mice (HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)AlbCre(+-)) and the HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) transgenic mice. Data on a limited number of these mice indicated that HBV transcription was largely unaffected by CCl$_4$ administration suggesting that fibrosis *per se* may not be directly mediating the
signals responsible for the loss of HBV biosynthesis in the FoxA-deficient HBV transgenic mice. However, more extensive fibrosis and additional characterization of this model is required to definitively establish the relationships between HBV biosynthesis, cytokine production, stellate cell activation, biliary epithelial cell proliferation and fibrosis.

Many studies have concluded that cytokine signaling can regulate HBV biosynthesis both at the transcriptional and post-transcriptional levels (49,96,98). It is possible that the inhibition of HBV biosynthesis in HBV(+-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) transgenic mice may result from the increased cytokine signaling in these mice. However, results presented in this study strongly indicate that a cytokine signaling mechanism cannot fully account for the inhibition of HBV biosynthesis observed. One finding supporting this conclusion is while there is inhibition of HBV biosynthesis in the 2 week old HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl) FoxA3(+-)AlbCre(+-) transgenic mice, there is no significant difference in cytokine signaling between the 2 week old HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) transgenic mice and the 2 week old cre negative HBV(+-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-) transgenic mice. Also preliminary analysis of FoxA-deficient HBV transgenic mice treated with peroxisome proliferators demonstrated an upregulation of HBV biosynthesis in only a few cells, potentially the few cells not deleted for FoxA (data not shown). This finding further supports the conclusion that a cytokine signaling mechanism cannot fully account for the inhibition of HBV biosynthesis.
5.4 Model

Figure 23 is a model depicting the progression of HBV transcriptional regulation knowledge attained owing to results presented in this study. Panel A represents the finding that in the absence of nuclear receptor expression vectors, the HBV 3.5 kb pregenomic RNA is not expresses and viral replication is not apparent in transient transfection analysis in non-hepatoma cells (Section 3.1.1). In contrast, panel B represents that multiple nuclear receptors are capable of activating HBV transcription and replication in the human embryonic kidney 293T cell line (Section 3.1.1).
Figure 23. **Model of HBV transcriptional regulation.** A-D) NR represents the nuclear receptors that have been demonstrated to activate HBV transcription and replication in non-hepatoma cells. PGC1α represents itself and other co-activators capable of augmenting the nuclear receptor mediated activation of HBV biosynthesis in cell culture. SHP represents itself and other potential co-repressors capable of inhibiting the nuclear receptor mediated activation of HBV biosynthesis in cell culture. E) NR represents the redundancy of nuclear receptor transcriptional regulation in HBV biosynthesis *in vivo*. FoxA represents the finding that HBV biosynthesis is inhibited when FoxA is deficient. PGC1α represents itself and other co-activators potentially activating HBV biosynthesis *in vivo*. 
HNF4α, RXRα/PPARα, RXRα/FXRα, LRH1, ERRβ and ERRγ, stimulate transcription of the HBV 3.5 kb RNA and this is associated with the synthesis of encapsidated viral replication intermediates (Fig 4). Moreover, this activation was demonstrated to function through the nucleocapsid promoter sequence (Fig 5). In cell culture these nuclear receptors are sufficient to activate HBV biosynthesis. The findings presented in this study indicate that under various physiological states, HBV biosynthesis could be regulated by different nuclear receptors and this redundancy of function as liver physiology changes might permit constant HBV biosynthesis. Panels C and D symbolize the competition between PGC1α (representing co-activators) and SHP (representing co-repressors) to regulate HBV biosynthesis in cell culture (Section 3.1.2). Furthermore, signals such as bile acids can affect this competition by activating both FXR and SHP, and thus, have only a modest effect on HBV biosynthesis in vivo (Section 3.2). Lastly, panel E represents the concept that HBV transcription is activated by multiple nuclear receptors and potentially PGC1α or other co-activators in vivo. Also HBV biosynthesis is significantly inhibited in FoxA-deficient HBV transgenic mice (Section 4.3). Therefore, results presented in this study suggest that FoxA plays a critical role in HBV transcription and thus HBV replication in vivo.

5.5 Future Directions

As deletion of the FoxA genes early in development by the use of the liver-specific AlbCre transgene might have as yet undetermined pleiotropic
effects, a future direction to further elucidate this decrease in HBV biosynthesis in the FoxA-deficient HBV transgenic mice is to delete the FoxA genes in the adult HBV transgenic mouse using the adenovirus vector, Adenovirus-Cre-GFP, expressing the Cre recombinase and GFP (Vector Biolabs). Infection of these mice with Adenovirus-Cre-GFP will delete the floxed FoxA alleles in these mice. These mice could be examined for adenovirus infection by GFP expression, while a decline in HBV biosynthesis might be correlated with the loss of FoxA expression and the development of fibrosis and cytokine and/or growth factor production over time. Critically, the Adenovirus-Cre-GFP would have to be highly efficient to lead to successful deletion of FoxA comparable to the HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) transgenic mice. This analysis might help resolve the remaining issues relating to the relative importance of FoxA deficiency compared to fibrosis development for the loss of HBV biosynthesis.

Furthermore, results presented in this study suggest that lack of FoxA itself may be leading to HBV inhibition. Perhaps FoxA is critical for nuclear receptor binding to the HBV core promoter. Therefore, although there is nuclear receptor redundancy to maintain HBV biosynthesis under many physiological circumstances, perhaps HBV biosynthesis is inhibited without FoxA since the nuclear receptors are not binding when FoxA is deleted. One way to assess this hypothesis would be to use chromatin immunoprecipitation (ChIP) to determine if there is a change in nuclear receptor binding to the HBV core promoter in HBV(+/−)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+/−)AlbCre(+/−) transgenic mice.
compared to cre negative HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-) transgenic mice. One could also use ChIP to determine if there are changes in recruitment of co-activators to the HBV core promoter in HBV(+-)FoxA1(fl/fl) FoxA2(fl/fl) FoxA3(+-)AlbCre(+-) transgenic mice compared to cre negative HBV(+-)FoxA1(fl/fl) FoxA2(fl/fl)FoxA3(+-) transgenic mice.

Another future direction would be to attempt to block cytokine signaling in the HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) transgenic mice using neutralizing antibodies. This experiment would allow examination of what contribution the cytokine signaling is having on the inhibition of HBV biosynthesis in the HBV(+-)FoxA1(fl/fl)FoxA2(fl/fl)FoxA3(+-)AlbCre(+-) transgenic mice.

5.6 **Summary**

In summary, HBV is a major human pathogen that currently chronically infects approximately 400 million individuals worldwide and is responsible for about one million deaths annually. New modalities of therapy are urgently needed to address this major public health issue. The results presented show multiple nuclear receptors are capable of activating HBV biosynthesis in non-hepatoma cells, including RXRα/FXRα, and both SHP and PGC1α can modulate RXRα/FXRα-dependent HBV biosynthesis in non-hepatoma cells. However, bile acids have only a modest effect \textit{in vivo}. Based on the cell culture analysis (Fig 11), it is possible that \textit{in vivo} even the activation of FXR by bile acids is not sufficient to further enhance HBV transcription and replication, which is being
directed by other constitutively active nuclear receptors. Of great interest, the results presented suggest that FoxA transcription factor deficiency *in vivo* can selectively prevent HBV biosynthesis without greatly altering hepatocyte physiology. However significant fibrosis, a common consequence of HBV infection, is apparent in the FoxA-deficient mice. It remains to be determined what the relative contributions from direct transcriptional regulation by FoxA and indirect effects mediated by nonparanchymal cells are to HBV biosynthesis in this system. Although findings presented in this study suggest FoxA deficiency itself can result in inhibition of HBV biosynthesis, it is quite possible both modes of regulation contribute to the observed effects on HBV biosynthesis.
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Vanessa Reese  
E-Mail: vreese2@uic.edu

Education

University of IL at Chicago, Medical Scientist Training Program (MSTP)  
MD. /PhD. Candidate, expected graduation in 2014  
June 2006-Present

Brown University, BA  
Concentration: Community Health, graduated with honors, GPA of 3.9 within concentration  
Sept 2002-May 2006

Research and Work Experience

PhD research in Dr. McLachlan’s lab on regulation of Hepatitis B Virus transcription  
Summer 2008 to present
  Defended April 2012

Research at RI Medical Examiners’ Office and Brown University  
Assessing drug-related deaths of undetermined intent  
Fall 2005 to Spring 2006

Summer Internship Program at Harvard School of Public Health  
Conducted research in Dr. Jay Mizgerd’s lab on Streptococcus vs E. coli caused pneumonia  
Summer 2005

MSTP SURF Program at UC San Diego  
Summer 2004

Cochrane Center at Brown University  
Handsearching and processing of CENTRAL submissions  
Jan 2004 to Sept 2005

Summer Medical Education Program at Yale School of Medicine  
Summer 2003

Teaching Experience

MCAT Course Instructor, Kaplan Test Prep  
Prepared and instructed classroom courses on all subject areas tested on the MCAT; Positions only offered to those who score above the 90th percentile on the MCAT; Also taught for PCAT and GRE  
Summer 2006 to Summer 2008

MCAT Personal Tutor, Kaplan Test Prep  
Prepared and delivered individualized instruction sessions to students for all MCAT subject areas; Positions only offered to those who score above the 90th percentile on the MCAT  
Summer 2006 to Summer 2008

TA for BI 28: Introduction to Biochemistry at Brown University  
Spring 2006

Brown Tutor for BI 28 (intro to Biochemistry) and BC 203 (grad level biostatistics)  
Fall 2005 to Spring 2006

TA for BC 132: Survey Research in Health Care at Brown University  
Spring 2005 and Spring 2006
Honors and Awards

NIH Fellowship - F31 Ruth L Kirschstein National Research Service Award – Individual Fellowship – awarded August 2009
Best Undergraduate Research Poster Presentation at Brown’s Public Health Research Day – April 5, 2005
Youth Foundation’s Hadden Scholarship for undergraduate education (awarded the max amount and renewed all 4 years) – 2002 to 2006
Awarded membership into Cum Laude Society at The Hill School – 2002
Yi-Cha Lee Math Award – 2002
George C. Whiteley, Jr. prize for interest and excellence in all sciences – 2002
Bausch + Lomb Science Award (in recognition of outstanding academic achievement and superior intellectual promise in the field of science – from U of Rochester) – 2001
Prudential Spirit of Community Distinguished Finalist 1998 for SC and Presidential Service Award Nominee 1997

Conferences and Presentations

Attended American Society for Clinical Oncology Annual Meeting – June 2013
Presented poster at American Society of Microbiology (ASM) 111th annual meeting – May 21-24, 2011
Attended American Physician Scientist Association (APSA) 7th Annual Meeting – April 15-17, 2011
Attended American Association for Cancer Research (AACR) 102nd Annual Meeting – April 2-6, 2011
Attended Translational Cancer Medicine (USA) 2010, AACR meeting – July 11-14, 2010
Presented summer research at Harvard School of Public Health – August 2005
Presented research in poster session at Brown’s Public Health Research Day – April 5, 2005
Presented research at UCSD conference – August 12, 2004

Publications


