Chapter 3
The Regulation of HBV Transcription and Replication

Claudia E. Oropeza, Grant Tarnow, Abhayavarshini Sridhar, Taha Y. Taha, Rasha E. Shalaby, and Alan McLachlan

Abstract Hepatitis B virus (HBV) is a major human pathogen lacking a reliable curative therapy. Current therapeutics target the viral reverse transcriptase/DNA polymerase to inhibit viral replication but generally fail to resolve chronic HBV infections. Due to the limited coding potential of the HBV genome, alternative approaches for the treatment of chronic infections are desperately needed. An alternative approach to the development of antiviral therapeutics is to target cellular gene products that are critical to the viral life cycle. As transcription of the viral genome is an essential step in the viral life cycle, the selective inhibition of viral RNA synthesis is a possible approach for the development of additional therapeutic modalities that might be used in combination with currently available therapies. To address this possibility, a molecular understanding of the relationship between viral transcription and replication is required. The first step is to identify the transcription factors that are the most critical in controlling the levels of HBV RNA synthesis and to determine their in vivo role in viral biosynthesis. Mapping studies in cell culture utilizing reporter gene constructs permitted the identification of both ubiquitous and liver-enriched transcription factors capable of modulating transcription from the four HBV promoters. However, it was challenging to determine their relative importance for viral biosynthesis in the available human hepatoma replication systems. This technical limitation was addressed, in part, by the development of non-hepatoma HBV replication systems where viral biosynthesis was dependent on complementation with exogenously expressed transcription factors. These systems revealed the importance of specific nuclear receptors and hepatocyte nuclear factor 3 (HNF3)/forkhead box A (FoxA) transcription factors for HBV biosynthesis.

C. E. Oropeza · G. Tarnow · A. Sridhar · T. Y. Taha · A. McLachlan (✉)
Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA
e-mail: mclach@uic.edu

R. E. Shalaby
Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA

Department of Microbiology and Immunology, Faculty of Medicine, Tanta University, Egypt, Egypt

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Furthermore, using the HBV transgenic mouse model of chronic viral infection, the importance of various nuclear receptors and FoxA isoforms could be established in vivo. The availability of this combination of systems now permits a rational approach toward the development of selective host transcription factor inhibitors. This might permit the development of a new class of therapeutics to aid in the treatment and resolution of chronic HBV infections, which currently affects approximately 1 in 30 individuals worldwide and kills up to a million people annually.

1 Introduction

Hepatitis B virus (HBV) infects man and great apes [1–11]. Viral tropism is restricted to the hepatocytes within the liver of the host [12–17]. HBV biosynthesis within the liver is noncytopathic [17–19]. However, the cellular immune response to HBV antigens synthesized during infection and presented at the cell surface of these hepatocytes in the context of human leukocyte antigens (HLA) results in cell death by T-cell-mediated cytotoxicity, compensating liver regeneration and associated fibrosis [18, 19]. In long-term chronic carriers where these processes have occurred for many years, cirrhosis and end-stage liver diseases can occur [18, 19]. Furthermore, chronic HBV carriers are at much greater risk of developing hepatocellular carcinoma (HCC) [18–21]. Liver cirrhosis and HCC are associated with significant morbidity and mortality [22]. It is estimated that approximately one in three individuals in the world will be infected with HBV in their lifetime, resulting in about 1 in 30 individuals currently being chronic carriers [23, 24]. This translates into approximately 248 million chronic HBV carriers worldwide today and an associated yearly mortality due to HBV-associated disease of about 600,000 individuals [22–24]. Therefore, HBV is a major public health concern, which currently lacks any therapies capable of efficiently resolving chronic infection [25, 26]. Current therapies are limited to type 1 interferons and nucleoside analog drugs, which modulate the immune response and inhibit the HBV reverse transcriptase/DNA polymerase, respectively [25, 26]. As these long-term therapies are generally used to limit disease progression [25, 26], there is an urgent need for additional therapeutic modalities capable of resolving chronic HBV infections within a limited treatment time period.

2 Transcription of the HBV Genome

The cloning and sequencing of HBV genomic DNA identified four open reading frames within the viral 3.2kbp genome [27–30]. Here, the sequence coordinates of the HBV*ayw* subtype (genotype D [27, 31]) will be used, but the overall genome organization is essentially identical for all replication-competent viral genomes despite modest nucleotide and amino-acid differences among the various genotypes (subtypes) [27–30]. The core or nucleocapsid open reading frame encodes the hepatitis B early and core antigens, HBeAg and HBcAg, respectively (Fig. 3.1) [1, 32]. HBeAg is synthesized from the first translation initiation codon of the nucleocapsid

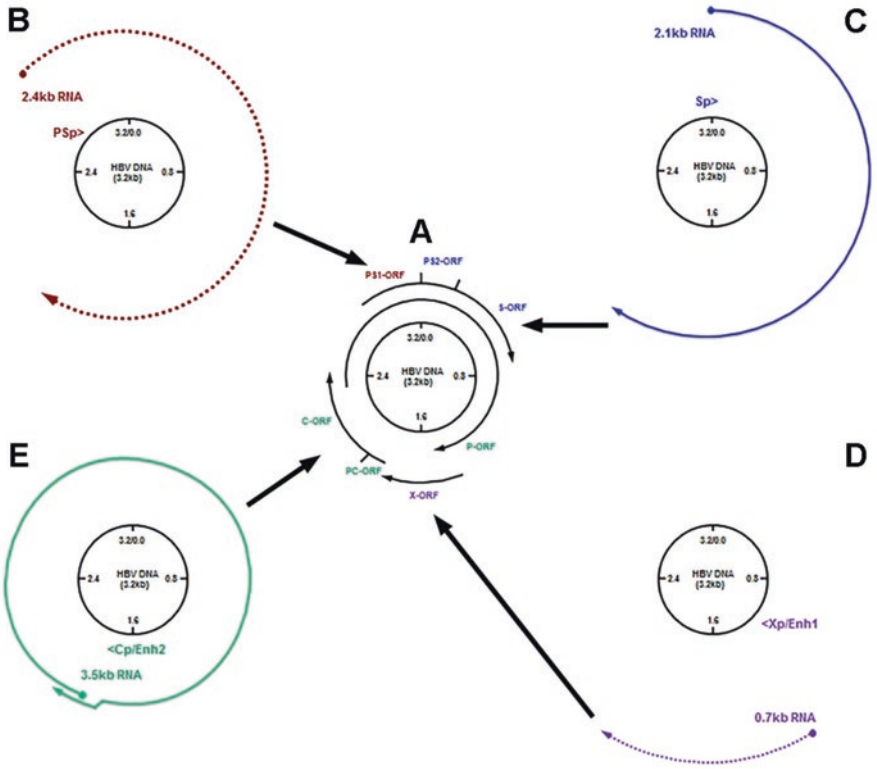


Fig. 3.1 Organization of the HBV genome. The circular HBV genome (subtype ayw) is 3182 nucleotides in length. The position of nucleotide coordinates 800 (0.8), 1600 (1.6), 2400 (2.4), and 3182 (3.2/0.0) are indicated. (*A*) The viral open reading frames (ORFs) are represented by black arrows. Orientation is N terminal to C-terminal for the PS (presurface), S (surface), X (X-gene), PC (precore), C (core), and P (polymerase) ORFs. The direction of transcription (>) from (*B*) the large surface antigen promoter (PSp), (*C*) the major surface antigen promoter (Sp), (*D*) the enhancer 1/X gene promoter (Enh1/Xp), and (*E*) the enhancer 2/core or nucleocapsid promoter (Enh2/Cp) is shown. Abundant 3.5-kb and 2.1-kb HBV transcripts are indicated by the solid green and blue arrows and the relatively rare 2.4-kb and 0.7-kb transcripts are indicated by the broken brown and purple arrows, respectively. The four transcripts terminate at the single polyadenylation site located around nucleotide coordinate 1940

212 amino-acid open reading frame [33–37]. The first 19 amino-terminal hydrophobic signal sequence residues are cleaved by the signal peptidase as the precore sequence is translocated into the endoplasmic reticulum [33, 36–39]. Subsequently, the 34 carboxyl-terminal arginine-rich nuclear localization sequence residues are cleaved from the HBeAg precursor by a furin protease in the Golgi apparatus [33, 40–42]. This results in the secretion of a 36 kDa HBeAg protein comprising a dimer of the 159 amino-acid polypeptide generated as a result of the amino- and carboxyl-terminal cleavage events of the product of the complete nucleocapsid open reading frame [43, 44]. The 21 kDa HBcAg polypeptide is synthesized from the second in-frame translation initiation codon of the nucleocapsid open reading frame, which can assemble to generate the viral capsid comprising 120 dimers [33, 45–48].

The surface antigen open reading frame encodes the viral envelope proteins (Fig. 3.1) [1, 27, 33]. There are three in-frame translation initiation codons within this open reading frame, which are translated to produce the large, middle, and major surface antigen proteins, HBsAg [1, 27, 33]. The large surface antigen protein, p39/gp43, includes the 108 amino acid preS1, 55 amino acid preS2, and 226 amino acid major surface antigen domains, whereas the middle surface antigen protein, gp33/gp36, includes only the pres2 and major surface antigen domains [33, 49, 50]. The major surface antigen, p25/gp28, is translated from the third initiation codon and encodes the carboxyl-terminal 226 amino acids of the surface antigen open reading frame [33, 51–53]. All three HBsAg translation products are partially glycosylated at asparagine 146 of the major surface antigen open reading frame, whereas asparagine 4 of the pres2 domain present in the middle surface antigen polypeptide is completely glycosylated [54–57]. This gives rise to the six different forms of the HBsAg polypeptide present in the virus particles [58].

The HBV viral genome encodes two additional open reading frames. The HBV reverse transcriptase/DNA polymerase open reading frame encodes a 94 kDa polypeptide with three major domains (Fig. 3.1) [27]. The amino-terminal domain of this open reading frame encodes the terminal protein, which serves as the primer for HBV minus-strand DNA synthesis [59–63]. The middle domain encodes the reverse transcriptase/DNA polymerase activity, while the carboxyl-terminal domain encodes for the RNaseH activity responsible for the degradation of the viral pregenomic RNA during the process of minus-strand DNA synthesis [64–71]. The smallest open reading frame in the viral genome codes for a 154 amino-acid polypeptide, HBxAg (Fig. 3.1) [27]. The 17 kDa X-gene open reading frame encodes a protein that is essential for productive viral infection in vivo and has been ascribed a large variety of functions when assayed under various conditions [72–75]. Currently, it is unclear which, if any, of these functions explains the requirement for this protein for productive infection in vivo.

Analysis of the HBV viral transcripts during natural infection of humans and chimpanzees has been modest due to the limited availability of liver samples. However, two predominant viral transcripts of 3.5 kb and 2.1 kb have been detected during natural infection (Fig. 3.1) [12–16]. Furthermore, analysis of viral transcripts present in cells transfected with HBV genomic DNA and HBV transgenic mice has permitted a more detailed analysis of the transcripts derived from viral genomes. In addition to the major transcripts, two additional unspliced HBV RNAs of 2.4 and 0.7 kb have been routinely described in a variety of systems that can support viral biosynthesis (Fig. 3.1) [76–89]. The 3.5 kb HBV transcripts identified by RNA filter hybridization analysis represent two distinct transcripts, the precore and pregenomic RNAs, as determined by 5′-end mapping studies, which differ by approximately 36 nucleotides (Fig. 3.2) [76–78, 90]. The 3.5 kb HBV precore RNA initiates at a cluster of sites centered at approximately nucleotide coordinate 1785 and its translation from the initiation codon at nucleotide 1816 results in the synthesis of HBeAg [76–78, 90]. The 3.5 kb HBV pregenomic or core RNA initiates at a cluster of sites centered at approximately nucleotide coordinate 1821 and its translation from the initiation codon at nucleotide 1903 results in the synthesis of HBcAg [76–78, 90]. The 3.5 kb HBV pregenomic RNA is also translated from an internal initiation

codon at nucleotide 2309, which results in the synthesis of the viral reverse transcriptase/DNA polymerase polypeptide although this presumably occurs at a much lower frequency than translation of the HBcAg polypeptide [65, 91, 92]. In this manner, the structural HBcAg is synthesized at a level much greater than the viral polymerase, which supports efficient viral biosynthesis. Furthermore, the HBV polymerase recognizes the RNA stem/loop/bulge structure, epsilon (ε), at the 5′-end of the 3.5 kb pregenomic RNA as it is being translated from the ribosome and forms a ribonucleoprotein complex, which is encapsidated by HBcAg to generate immature core particles [69, 93–95]. Within these immature core particles, the viral polymerase reverse transcribes the 3.5 kb pregenomic RNA to generate the mature core particle containing the 3.2 kb relaxed circular HBV DNA genome [93, 94]. Mature core particles can bind to envelope antigen, HBsAg, located within the membrane of the endoplasmic reticulum and subsequently bud into the lumen to be secreted from the hepatocytes by transit through the Golgi apparatus [96–101]. Alternatively, mature capsids can cycle viral genomes back into the nucleus to amplify and/or replenish the pool of HBV covalently closed circular DNA (HBV cccDNA) that represents the template for transcription by the host RNA polymerase II [79, 102].

The 2.1 kb HBV transcripts identified by RNA filter hybridization analysis appear to initiate synthesis at a cluster of locations positioned between nucleotide coordinates 3156 and 8, as determined by 5′-end mapping studies (Fig. 3.2) [76–78, 103]. As a consequence of the heterogeneous nature of the transcription start sites and their proximity to the preS2 initiation codon at nucleotide coordinate 3174, the 2.1 kb HBV surface antigen RNA is translated to a rather modest degree from the preS2 initiation codon at nucleotide coordinate 3174 to produce limited amounts of the middle HBsAg polypeptide and is robustly translated from the initiation codon at nucleotide coordinate 157 to produce large quantities of the major surface antigen protein [76–78, 103]. The minor 2.4 kb HBV presurface RNA initiates at a cluster of sites centered at approximately nucleotide coordinate 2809 and its translation from the initiation codon at nucleotide 2850 results in the synthesis of a limited amount of the large surface antigen polypeptide [76–78]. Consequently the large, middle, and major HBsAg polypeptides are synthesized at appropriate ratios to support the synthesis of virus particles, which require the large surface antigen polypeptide, plus orders of magnitude more subviral particles, which are present in the sera of infected individuals [58]. The 0.7 kb HBV X-gene RNA, which has been observed in some cell culture systems, HBV transgenic mice, and infected liver tissues, appears to initiate at multiple sites spanning nucleotide coordinates 1157 and 1340 and its translation from the initiation codon at nucleotide 1376 could result in the synthesis of the HBV X-gene polypeptide [89, 104–106]. The X-gene-encoded protein product has not been convincingly demonstrated in natural infection although antibodies to this polypeptide have been detected in the sera of chronic HBV carriers [89, 107]. Therefore, it is not apparent if the HBV X-gene polypeptide is encoded by its own transcript during natural infection, translated from one or more of the larger HBV RNAs by internal ribosome entry, or translated from a minor spliced HBV transcript. For all of the HBV transcripts, polyadenylation of the viral RNAs occurs between nucleotide coordinates 1936 and 1943, mediated in

part by the nonconventional polyA recognition sequence, 5′-UAUAAA-3′, located between nucleotide coordinates 1918 and 1923 [85, 103].

3 *Cis*-Acting Transcriptional Regulatory Sequence Elements and *Trans*-Acting DNA-Binding Proteins

The cloning of the HBV genome and the mapping of the transcripts suggested that there were likely to be four transcriptional regulatory regions controlling viral RNA synthesis. With the extensive use of reporter gene constructs and transfection analysis utilizing both hepatoma and nonheptoma cell lines, the *cis*-acting transcriptional regulatory sequence elements within the viral genome were mapped in detail by deletion and mutational analysis (Fig. 3.2). Sequences of 70–240 nucleotides

Fig. 3.2 (continued) receptor–binding site direct repeat sequence 5′-AGGTCA-3′ are indicated with arrows. The underlined sequences in the enhancer 1/X-gene promoter region indicate the location of the CCAAT/enhancer-binding protein-binding sites (C/EBP) [139], the p53 tumor suppressor gene product–binding site (p53) [140], the interferon regulatory factor–binding site (IRF) [141], the nuclear factor 1–binding sites (NF1) [142, 143], the forkhead box protein A/hepatocyte nuclear factor 3–binding sites (FOXA/HNF3) [144, 145], the hepatocyte nuclear factor 4–binding site (HNF4) [127], the retinoid X receptor plus the peroxisome proliferator-activated receptor heterodimer–binding site (RXR:PPAR) [127, 128, 146], the COUPTF-binding site (COUPTF) [120, 127], the RFX1-binding site (RFX1) [127, 147, 148], the activator protein 1–binding site (AP1) [143], the cyclic AMP response element–binding protein-binding site (CREB) [149], and the activating transcription factor 2–binding site (ATF2) [149]. The underlined sequences in the enhancer 2/core promoter region represent the RFX1-binding site (RFX1) [108], the Sp1-binding sites (Sp1) [109], the CCAAT/enhancer-binding protein-binding site (C/EBP) [110, 111], the retinoid X receptor plus the farnesoid X receptor heterodimer–binding site (RXR:FXR) [115–118], the liver receptor homolog 1/fetoprotein transcription factor–binding sites (LRH1/FTF) [112–114], the hepatic leukemia factor–binding site (HLF) [113], the E4BP4-binding site (E4BP4) [119], the hepatocyte nuclear factor 4–binding sites (HNF4) [120, 121], the forkhead box protein A/hepatocyte nuclear factor 3–binding sites (FOXA/HNF3) [122], the retinoid X receptor plus the peroxisome proliferator-activated receptor heterodimer–binding site (RXR:PPAR) [120], the COUPTF binding site (COUPTF) [120, 123, 124], the estrogen-related receptor (ERR) [117, 125], and the TATA-box-binding protein (TBP) site [126]. The underlined sequences in the intragenic core gene region spanning nucleotide coordinates 2110 to 2200 sequence indicate the location of the Sp1-binding sites (Sp1), the forkhead box protein A/hepatocyte nuclear factor 3–binding site (FOXA/HNF3), and the hepatocyte nuclear factor 4–binding site (HNF4). The underlined sequences in the large surface antigen promoter region indicate the location of the hepatocyte nuclear factor 1–binding sites (HNF1) [129, 130], the forkhead box protein A/hepatocyte nuclear factor 3–binding site (FOXA/HNF3) [131], the Sp1-binding sites (Sp1) [132], and the TATA-box-binding protein (TBP) site [133]. The underlined sequences in the major surface antigen promoter region indicate the location of the forkhead box protein A/hepatocyte nuclear factor 3–binding sites (FOXA/HNF3) [134], the nuclear factor 1–binding site (NF1) [135, 136], the Sp1-binding sites (Sp1) [137], and the nuclear factor Y–binding site (NF-Y) [138]. The approximate positions of the major transcription start sites are indicated by solid circles plus arrows indicating the direction of transcription. The transcription polyadenylation signal sequence, 5′-UAUAAA-3′, and the sights of polyadenylation for the viral RNAs are indicated with open and closed boxes, respectively. The protein translation initiation codons for the seven HBV polypeptides are indicated with solid triangles

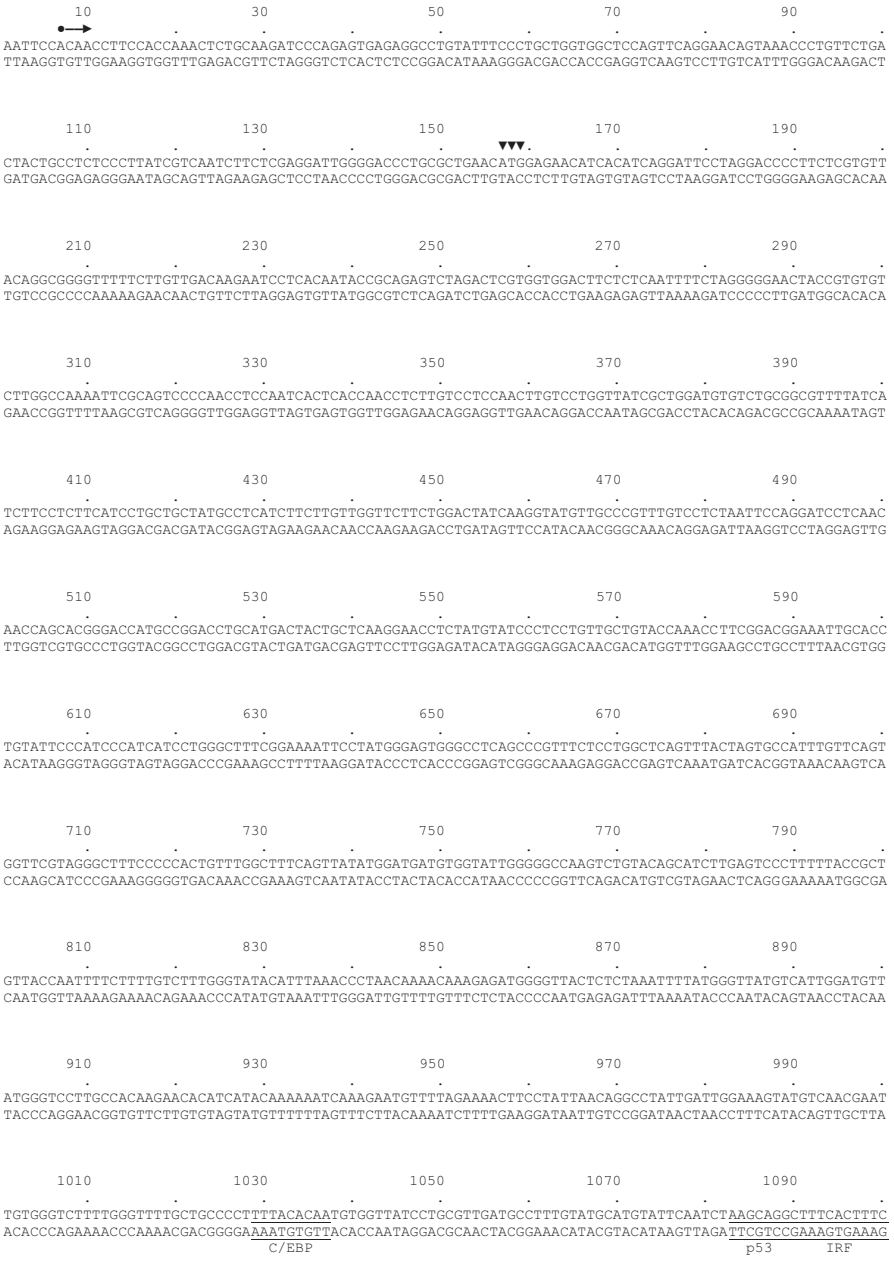
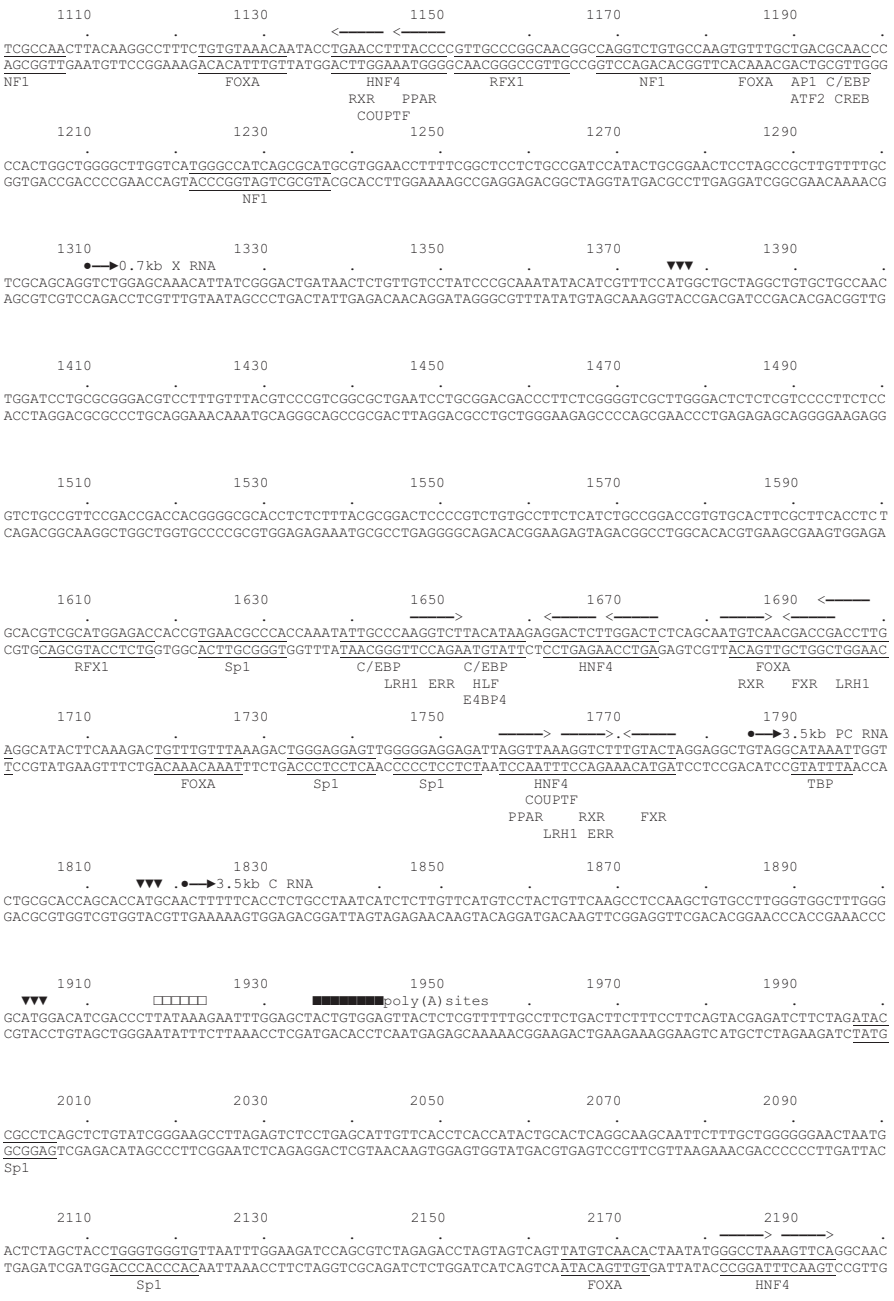


Fig. 3.2 Nucleotide sequence of the HBV genomic DNA (subtype *ayw*) showing the location of the transcription factors binding to the enhancer 1/X-gene promoter, enhancer 2/core promoter region, the intragenic core gene sequence, the large surface antigen promoter, and the major surface antigen promoter [27]. The nucleotide coordinates are derived from the GenBank database (ID: V01460). The orientation of the direct repeat sequences homologous to the consensus nuclear



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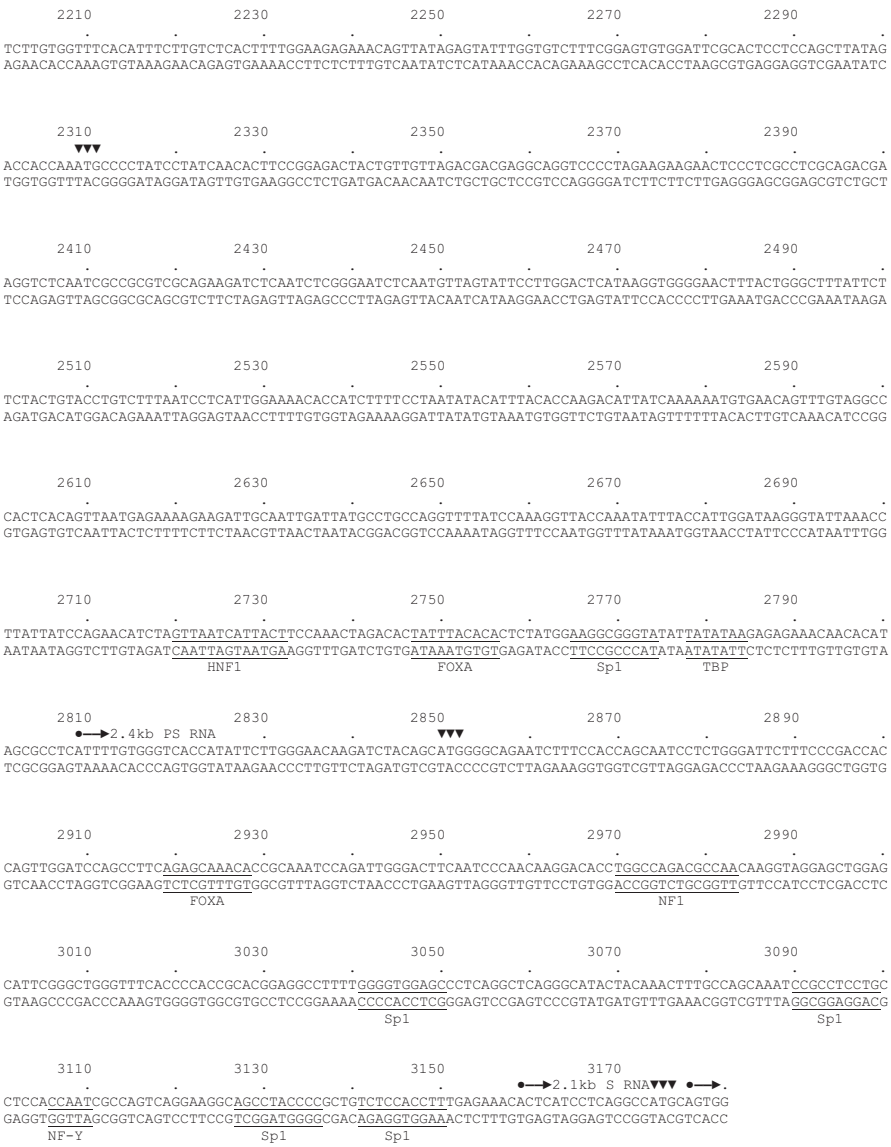

Fig. 3.2 (continued)

located upstream of the transcription start sites for each of the viral transcripts were shown to correspond to the enhancer/promoter regions governing the levels of RNA synthesis. The 200 base pair region located between nucleotide coordinates 1600 and 1800 bound both ubiquitous (RFX1, SP1, COUPTF, ERR, and TBP) and liver-enriched (C/EBP, LRH1, HNF4, RXR, FXR, FOXA and PPAR) transcription factors, which contributed to the level of the nucleocapsid or core promoter activity that directs the expression of the HBV 3.5 kb precore and pregenomic RNAs encoding the HBeAg and HBcAg polypeptides [108–128]. The 70 base pair region located between nucleotide coordinates 2720 and 2790 bound both ubiquitous (SP1 and TBP) and liver-enriched (FOXA and HNF1) transcription factors, which contribute to the level of the presurface antigen promoter activity that directs the expression of the HBV 2.4 kb presurface antigen RNA encoding the large surface antigen [129–133]. The 240 base pair region located between nucleotide coordinates 2910 and 3150 bound both ubiquitous (NF1, SP1 and NF-Y) and liver-enriched (FOXA) transcription factors, which contributed to the level of the surface antigen promoter activity that directs the expression of the HBV 2.1 kb surface antigen RNAs encoding the middle and major surface antigens [134–138]. Similarly, the 220 base pair region located between nucleotide coordinates 1020 and 1240 bound both ubiquitous (p53, IRF, NF1, COUPTF, RFX1, AP1, CREB, and ATF2) and liver-enriched (C/EBP, FOXA, HNF4, RXR and PPAR) transcription factors, which contributed to the level of the X-gene promoter activity that may direct the expression of the HBV 0.7 kb X-gene RNAs encoding the X-gene polypeptide [120, 127, 139–149]. Furthermore, the X-gene and core promoter regions can act as enhancer sequences, enhancer 1 and 2, respectively, under certain circumstances leading to increased transcription from the other HBV promoters [104, 112, 113, 145, 146, 150–168]. The enhancer function of the X-gene and core promoter regions may be important for the coordinated liver-specific expression of the HBV transcripts. Similarly, the contribution of individual transcription factors to multiple HBV promoter activities may also control the coordinate expression of the various transcripts at levels appropriate for viral biosynthesis. For example, FOXA binds to and regulates expression from all four HBV promoters to various extents [131]. However, the large surface antigen promoter is considerably weaker than the other HBV promoters due, in part, to its limited number of transcription factor–binding sites [166]. This ensures that limiting amounts of the large surface antigen are synthesized and hence prevents the inhibition of viral secretion due to the overproduction of surface antigen tubules that can limit viral envelope secretion [33, 49, 169].

The mapping of the *cis*-acting promoter sequences permitted the identification of regulatory sequence elements that were transcriptionally active only in hepatoma cells and not in nonhepatoma cells [87, 90, 105, 136, 150, 151, 158, 159, 164, 166, 167, 170–180]. These regulatory elements bound liver-enriched transcription factors, whereas the promoter regulatory elements that were transcriptionally active in both cell types bound more ubiquitously expressed transcription factors. Combinations of DNaseI footprinting and electrophoretic mobility shift assays (EMSAs) using cell extracts and purified factors permitted the identification of many of the *trans*-acting transcription factors binding to the HBV promoter regulatory

sequence elements [109, 120–122, 124, 129–134, 136, 137, 150, 157, 161, 165, 170, 177, 178, 181–186]. Functional validation of the roles of the identified DNA binding proteins in governing the activities of the HBV promoter was evaluated using transfection analysis of wild-type and mutant HBV reporter gene constructs in the presence of exogenously expressed transcription factors [109, 120, 121, 124, 129, 131, 133, 137, 157, 178, 183, 184, 186, 187]. These studies led to a relatively comprehensive map of the HBV enhances/promoters and the functional importance of the transcription factors that bind to these regulatory sequence elements (Fig. 3.2). Despite generating a relatively comprehensive map of the *cis*-acting regulatory sequence elements governing viral RNA synthesis and the *trans*-acting factors that bound to them, it remained unclear what the relative importance of the various identified transcription factors might be for HBV biosynthesis, either in cell culture or in vivo.

4 Role of Liver-Enriched Transcription Factors in HBV Transcription, Replication, and Tissue Tropism

The mapping of transcription factor–binding sites to the viral promoters permitted their role in controlling HBV RNA synthesis to be evaluated in the context of viral replication. A significant limitation of these studies arose, because robust viral replication could only be observed in a very limited number of hepatoma cell lines where all the necessary factors for HBV biosynthesis were present [76, 80, 82, 188]. This meant that the effects of exogenously expressed transcription factors on viral transcription were typically rather modest [115, 189]. Furthermore, it was challenging to map these modest effects to specific transcription factor–binding sites by mutational analysis, in part, because of the redundancies in the transcriptional regulation of HBV RNA synthesis and the potential effects of mutations on the viral-coding capacity or *cis*-acting sequences involved in the regulation of viral replication. The use of short interfering RNA (siRNA) technologies to reduce specific transcription factor abundances in hepatoma cells also has limited utility because of the functional redundancies in the DNA-binding proteins regulating HBV biosynthesis. For these reasons, it became necessary to develop additional approaches to study the effects of specific transcription factors on HBV RNA synthesis, and consequently viral replication.

HBV does not replicate in nonhepatoma cells, presumably because these cells lack the specific transcription factors necessary to support the synthesis of the 3.5 kb RNA from the viral core promoter [189, 190]. The suggestion was supported by the observation that viral 3.5 kb pregenomic RNA synthesis driven by the cytomegalovirus (CMV) immediate early promoter was sufficient to support robust HBV replication in nonhepatoma cells [191]. These findings suggested that complementation of HBV genomic DNA with the appropriate liver-enriched transcription factors in nonhepatoma cells represented an approach to identifying the roles of specific transcription factors in the synthesis of HBV 3.5 kb pregenomic RNA and hence viral replication [189]. Indeed, this approach identified nuclear receptors as the sole class of transcrip-

tion factors capable of robustly activating viral RNA and DNA syntheses in nonhepatoma cells [117, 189]. This approach indicated that HNF4, RXR, PPAR, FXR, and LRH1 represented liver-enriched nuclear receptors capable of supporting viral biosynthesis in nonhepatoma cells and hence likely contributed in a significant manner to the hepatocyte-specific tropism of HBV [117, 189]. The suggestion that HBV tropism is determined at the level of HBV 3.5 kb pregenomic RNA transcription is strongly supported by the tissue-specific expression pattern observed in the HBV transgenic mouse model of chronic viral infection [17]. In this model, viral transcription and biosynthesis are largely restricted to tissues where these transcription factors are highly expressed with lower levels of transcription being observed in tissues where these transcription factors are expressed at more modest levels [17, 192].

The development of the nonhepatoma cell system for the analysis of the transcriptional regulation of HBV biosynthesis identified nuclear receptors as the only transcription factors capable of supporting viral biosynthesis in this system [117, 189]. Furthermore, most of the activity was shown to map to the proximal nuclear receptor binding site located within the core promoter region [117, 189]. However, it was unclear what the role of the other liver-enriched transcription factors known to bind to the viral promoters might be in governing HBV transcription and replication. To date, none of the other liver-enriched transcription factors, except FoxA/HNF3, have been shown to modulate HBV biosynthesis in nonhepatoma cells [189]. In the nonhepatoma cell viral biosynthesis system, FoxA/HNF3 has been shown to antagonize nuclear receptor–mediated HBV transcription and replication [189, 191]. FoxA-/HNF3-mediated reduction in viral biosynthesis involves both HBeAg-mediated inhibition of HBV biosynthesis, possibly by reducing the efficiency of capsid assembly, plus inhibition of RNA elongation presumably by interfering with RNA polymerase II transcription through the viral promoters located within the DNA regions encoding the HBV 3.5 kb pregenomic RNA [191].

5 Redundant Functions for Nuclear Receptors in HBV Biosynthesis

The use of the nonhepatoma cell system permitted the identification of multiple nuclear receptors capable of supporting HBV biosynthesis due to their ability to bind to the viral nucleocapsid promoter and direct the expression of the HBV 3.5 kb pregenomic RNA [117, 189]. These observations may, in part, explain the difficulties in determining which transcription factors contribute most to HBV transcription in hepatoma cells and hepatocytes in vivo [118, 125, 193–195]. Differentiated hepatoma cells express a variety of liver-enriched transcription factors and support HBV transcription and replication [76, 80, 82, 188]. Consequently, ectopic expression of liver-enriched transcription factors can only enhance HBV transcription to a modest extent [115, 189]. Furthermore, reduction or elimination of any specific transcription factor involved in HBV RNA synthesis also only has a very modest effect as there are additional transcription factors capable of substituting for the loss of any particular

transcription factor [196]. This situation has also been observed in vivo where individual nuclear receptor-null HBV transgenic mice have displayed only modest perturbations in HBV biosynthesis. Specifically, the PPARα-null HBV transgenic mice displayed wild-type levels of HBV RNA and DNA under control conditions but failed to show enhanced biosynthesis when challenged with PPARα agonists [192, 193]. In contrast, liver-specific HNF4-null HBV transgenic mice displayed a complete loss of viral biosynthesis, indicating that this nuclear receptor was a major determinant of the developmental expression of HBV RNA [195]. However, early neonatal loss of HNF4 expression affects the abundance of additional nuclear receptors (and liver-enriched transcription factors), which are potentially critical for robust HBV RNA synthesis [197], making it unclear the degree to which HNF4 plays a direct or indirect role in the developmental regulation of HBV expression [195].

FXR has also been implicated in the regulation of HBV biosynthesis [115, 117, 118]. However, treatment of HBV transgenic mice with bile acids has only a very limited effect on viral biosynthesis [196]. This effect was not dependent upon inhibition by the corepressor, small heterodimer partner (SHP), as SHP-null HBV transgenic mice have a similar phenotype to their wild-type controls whether or not they were fed a diet supplemented with bile acids [196]. The redundant function of multiple nuclear receptors may explain these observations [117, 196]. In the nonhepatoma cell system, HNF4 and FXR are both capable of independently activating HBV biosynthesis [117]. In the presence of HNF4, FXR can only modestly modulate HBV biosynthesis accounting for the in vivo observations [196]. Therefore, the development of the nonhepatoma cell–based HBV replication system has permitted the reconstitution of viral biosynthesis and the demonstration of the redundant mechanisms, which probably operate in vivo to govern the level of viral transcription under different physiologically relevant conditions [196, 198].

6 Regulation of HBV Biosynthesis by Transcriptional Coactivators and Corepressors

The development of the nonhepatoma cell HBV replication system permitted a more detailed investigation of the potential roles of coactivator and corepressor proteins in the regulation of HBV transcription and replication [118, 125, 194, 199–201]. These studies demonstrated that the coactivators, PGC1α, CBP, SRC1, and PRMT1, and the corepressor, SHP, were all capable of modulating HBV transcription to some degree depending on cellular context [118, 125, 194, 199, 200]. Furthermore, the observation that PGC1α and SHP could modulate the nuclear receptor–dependent HBV biosynthesis in nonhepatoma cells further indica ted the potential importance of various nuclear receptors in the transcriptional regulation of viral biosynthesis [118, 125, 194, 199, 200].

Examination of PGC1α-dependent HBV biosynthesis in the nonhepatoma cell line, HEK293T, in the absence of exogenously expressed nuclear receptors revealed two major aspects of the transcriptional regulation of viral DNA synthesis [200].

First, PGC1α acted as an adapter molecule for the recruitment of additional coactivators in the absence of exogenously expressed nuclear receptors in this particular cell line [200]. This indicated that the endogenous coactivators present in HEK293T cells that were unrelated to the PGC1 family of coactivators were unable to activate HBV 3.5 kb RNA synthesis independently of PGC1α [200]. Therefore, the recruitment of additional coactivators was PGC1-dependant and mutational analysis suggested that PGC1 was recruited to the HBV nucleocapsid promoter, at least in part, through endogenous nuclear receptors present in HEK293T cells [200]. In addition to serving as an adaptor molecule for the recruitment of additional coactivator proteins, PGC1α enhanced HBV transcription in HEK293T cells such that these cells could now support robust viral replication [200]. Detailed analysis of the mechanism governing this observation demonstrated that the concentration of HBcAg passed a critical threshold necessary for core dimers to cooperatively form viral capsids (Fig. 3.3) [200, 202, 203]. Therefore, this cell culture system demonstrated compelling evidence that very modest changes in HBV 3.5 kb pregenomic RNA synthesis that led to less than a two-fold increase in HBcAg were, nonetheless, associated with a dramatic increase in viral DNA synthesis [200]. This finding showed the absence of a linear relationship between core protein synthesis and capsid-associated HBV biosynthesis, which is a critical observation that should be considered when evaluating the transcriptional regulation of viral replication.

The observation that the activities of coactivators and corepressors, which were shown to modulate HBV biosynthesis, are responsive to changes in metabolic cellular states led to the suggestion that viral transcription and replication might be modulated by the physiological state of the cell [201]. Indeed, based on these types of observations, the term “metabolovirus” was suggested to describe the potential relationship between HBV biosynthesis and the metabolic state of the cell [204]. This suggestion is supported by the observations that PGC1α activity is enhanced in vivo by fasting [198, 205–208] and SHP activity is induced by bile acids [196, 209–211], demonstrating a direct relationship between metabolic challenges and coactivator and corepressor activities. However, despite these observations, there is very limited evidence linking metabolic perturbations to changes in specific coactivator- or corepressor-mediated changes in HBV biosynthesis in either hepatoma cell lines or animal models of chronic viral infection [116, 196, 198, 201]. This may reflect the lack of importance of this form of regulation of HBV biosynthesis or the presence of multiple compensating mechanisms that maintain the homeostatic regulation of viral RNA and DNA as the relative abundances of coactivators and corepressor activities change in response to altering physiological conditions.

7 Transcriptional Regulation of HBV Replication In Vivo

As HBV animal infection models are essentially limited to man, chimpanzees, and tree shrews [3, 4, 7, 212–215], a detailed understanding of the transcriptional regulation of HBV replication in vivo has been very challenging. None of the available models of HBV infection are suitable to investigate the in vivo relevance of the

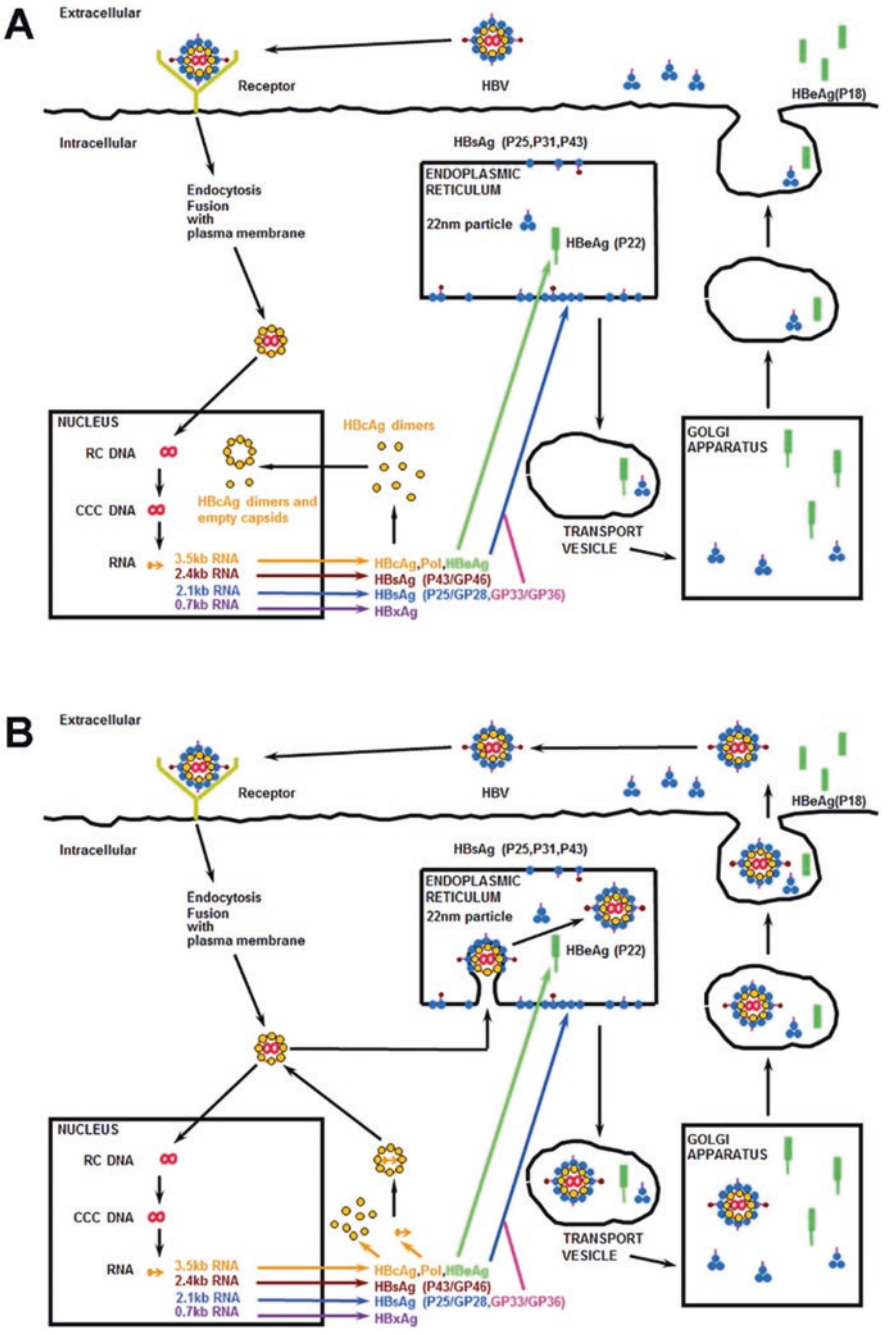

Fig. 3.3 The HBV replication cycle showing the intracellular pathway for the synthesis and secretion of HBV, HBsAg, and HBeAg polypeptides. (**a**) Lower levels of the HBV pregenomic 3.5 kb RNA preclude cytoplasmic dimer oligomerization, immature capsid formation, and hence HBV DNA synthesis. (**b**) Modestly higher levels of the HBV pregenomic 3.5 kb RNA permit cytoplasmic dimer oligomerization, immature capsid formation, and hence HBV DNA synthesis

transcriptional regulation of viral biosynthesis revealed in cell culture analysis. The small animal models of hepadnavirus infection including the woodchuck hepatitis virus (WHV) and the duck hepatitis B virus (DHBV) are also not useful models to understand the transcriptional regulation of HBV biosynthesis in vivo as the transcription of both WHV and DHBV is regulated in a distinct manner from HBV [216–218]. For all these reasons, the HBV transgenic mouse model of chronic HBV infection represents the most relevant and tractable small animal model for the study of the transcriptional regulation of HBV biosynthesis in vivo [17, 219]. In this model, a single replication competent HBV genome comprising 1.3 copies of the HBVayw DNA sequence has been incorporated into the mouse germline [17]. Consequently, every cell in the HBV transgenic mouse carries the viral transgene, which obviates the species barrier associated with viral infection. Furthermore, the HBV transgene is highly transcribed only in the tissues expressing the liver-enriched transcription factors identified in cell culture studies to control viral RNA synthesis [17, 192, 220]. Therefore, it appears that the HBV transgenic mouse model is a system that probably reflects quite closely the transcriptional regulation of HBV biosynthesis observed in the liver during natural infection. Furthermore, in the absence of any alternative in vivo model system for the investigation of the transcriptional regulation of HBV biosynthesis, it is appropriate to utilize this system to support findings in cell culture. As many of the observations in cell culture have been validated in the HBV transgenic mouse model, it is reasonable to assume that they probably reflect, in part, the situation in natural infection under certain circumstances.

A concern regarding the HBV transgenic mouse model was the absence of nuclear HBV cccDNA and hence the possibility that aspects of the viral life cycle in addition to infection were absent from this system [17]. The alternative explanation for the absence of HBV cccDNA was that cycling of capsids back to the nucleus was not preferred as a result of the high level of surface antigen expression and the large surface antigen in particular that is essential for capsid envelopment and virion secretion through the endoplasmic reticulum to the Golgi apparatus secretion system [221]. Based on this assumption, the HNF1α-null HBV transgenic mouse was created and analyzed [79, 222]. HNF1α regulates the level of expression from the large surface antigen promoter, so loss of HNF1α should be associated with a reduction in the level of HBV 2.4 kb RNA and hence translation of the large surface antigen polypeptide [129, 223]. This was predicted to lead to a reduction in virion production and the recycling of newly synthesized capsids to the nuclei [102, 224]. Interestingly, intracellular viral replication intermediates increased within the livers of HNF1α-null HBV transgenic mice despite very limited effects on HBV RNA synthesis [79]. Most interestingly, HBV cccDNA was readily apparent in these mice, demonstrating that recycling of capsids occurs in this HBV transgenic mouse model of chronic infection [79]. Furthermore, enhanced levels of viral replication were observed despite very limited changes in HBV transcription, supporting the contention that small changes in viral RNAs can be associated with large effects on DNA replication intermediates [79, 200].

As analysis in nonhepatoma cells indicated that nuclear receptors were major determinants of viral tropism, because they were critical for HBV 3.5 kb prege-

nomic RNA synthesis [189], it was of interest to determine their role in the in vivo regulation of HBV biosynthesis. Initially, PPARα-null HBV transgenic mice were characterized [193]. These mice displayed no major effect on HBV biosynthesis, indicating that PPARα did not contribute to viral transcription and replication under normal physiological conditions [193]. However, activation of PPARα by the agonists, clofibric acid and Wy-14,643, enhanced HBV biosynthesis in the liver of wild type but not PPARα-null HBV transgenic mice [193]. This finding demonstrated that activated PPARα can enhance the basal level of HBV biosynthesis observed in HBV transgenic mice [193]. As plasticizers and some drugs used to treat hypertriglyceridemia can activate PPARα, it seems possible that exposure to these compounds might affect viral loads and disease state of chronic HBV carriers due to their effects on viral biosynthesis [225, 226]. Furthermore, it was noted that the effect of PPARα activation in the HBV transgenic mouse activated viral DNA synthesis considerably more than RNA synthesis, suggesting that modest increases in transcription in vivo may be associated with much larger increase in viral replication as also recently observed in cell culture [193, 200].

Liver-specific HNF4α-null HBV transgenic mice died by postnatal day 15 [227]. The absence of HNF4α expression in the livers of these mice was associated with a dramatic loss in the increase in HBV biosynthesis observed during early neonatal development [227]. As HNF4α is a major contributor to the liver-specific transcriptional network that defines the hepatocyte phenotype [197], it is not clear if the effect of HNF4α on HBV biosynthesis is direct or indirect. However, the in vivo loss of HBV RNA and DNA synthesis associated with the absence of HNF4α expression is consistent with a direct role for this nuclear receptor in viral biosynthesis, as observed in the nonhepatoma replication system [189]. Furthermore, the observed increase in the developmental expression of HNF4α correlates with a similar developmental increase in HBV biosynthesis, supporting its potentially direct role in viral transcriptional regulation in vivo [189, 197]. However, the developmental expression HNF4α in the liver also supports the expression of additional transcription factors including LRH1, RXRα, FXRα, and FoxA2, which are also important regulators of HBV transcription and replication [189, 197]. If any of these transcription factors are critical determinants of viral biosynthesis, the effects of HNF4α on HBV RNA and DNA synthesis in vivo might be indirect rather than direct [189, 197].

Analysis of the liver-enriched transcription factors capable of complementing HBV transcription in nonhepatoma cells indicated that only nuclear receptors could independently activate HBV biosynthesis [117, 189]. This raised the interesting issue of the role of the other liver-enriched transcription factors in HBV biosynthesis in this system. Only FoxA/HNF3 modulated nuclear receptor-mediated biosynthesis in this system [189]. Indeed, it appeared that FoxA mediated its effects by preferentially reducing the expression of the HBV 3.5 kb pregenomic RNA at the level of transcriptional elongation, presumably due to its binding to the presurface, surface, X-gene and nucleocapsid promoters that are intragenic with respect to the transcription of the pregenomic RNA [191]. To address the in vivo relevance of these observations, HBV biosynthesis was determined in the liver-specific FoxA2/HNF3β-overexpressing HBV transgenic mouse [228]. As observed in the nonhepa-

toma cells, overexpression of FoxA2/HNF3β in the liver of the HBV transgenic mouse resulted in a dramatic reduction in HBV biosynthesis [228]. In this case, a large decrease in HBV replication was associated with a more modest reduction in viral transcription [228]. This observation suggests that the viral biosynthesis in the HBV transgenic mouse is positioned such than the small changes in HBV RNA synthesis result in limited effects on core polypeptide synthesis, which, due to the cooperative nature of capsid assembly, have a dramatic effect on capsid-dependent reverse transcription of pregenomic RNA in a manner similar to that recently reported in cell culture [200].

Since FoxA/HNF3 overexpression in the HBV transgenic mouse was associated with the loss of viral replication, it was of interest to determine the in vivo effect of the loss of FoxA/HNF3 on HBV biosynthesis [198, 220]. The FoxA3/HNF3γ-null HBV transgenic mouse displayed a very limited phenotype, suggesting that the other FoxA/HNF3 isoforms in the liver were either compensating for the loss FoxA3/HNF3γ or FoxA3/HNF3γ was relatively unimportant for HBV biosynthesis [198]. Consequently, a FoxA/HNF3-deficient HBV transgenic mouse expressing only a single FoxA3/HNF3γ allele was generated and characterized [220]. This mouse was viable and displayed no overt phenotype despite biliary epithelial cell proliferation, stellate cell activation, and bridging fibrosis within the liver [220, 229]. However, HBV transcription and replication were essentially absent within the liver [220]. Indeed, the HBV transgene had been permanently transcriptionally silenced due to DNA methylation of its non-CpG island sequences [220]. This observation indicated that the pioneer transcription factor, FoxA/HNF3, was essential for the demethylation of the HBV transgene during liver development and this may account, in part, for the observed increase in HBV biosynthesis during postnatal liver maturation [220, 227]. Further studies are required to determine when FoxA/HNF3 marks the HBV genome for demethylation during liver development and whether this process involves active demethylation by ten-eleven translocation (TET) methylcytosine dioxygenase-mediated oxidation of the 5-methylcytosine residues or passive demethylation involving DNA methyltransferase (DNMT) inhibition in the presence of chromosome replication [230]. Regardless of the mechanism of action of FoxA/HNF3, these observations suggest that targeting FoxA/HNF3 at the appropriate stage of liver development might lead to permanent DNA methylation and inactivation of HBV cccDNA as a transcriptional template necessary for viral biosynthesis and hence might represent a therapeutic target for the resolution of neonatal (and possibly adult) chronic infections.

8 Conclusions

HBV is a significant human pathogen responsible for approximately 600,000 deaths annually [22–24]. Current therapies are not curative and nucleoside-analog drugs target a single viral protein, the HBV reverse transcriptase/DNA polymerase, leading to the selection of drug-resistant variants [231, 232]. Additional

therapeutic targets are urgently needed to address this unmet need. Unfortunately, due to the small size of the viral genome and hence limited coding capacity, there are only a very limited number of HBV proteins that might serve as potential additional targets for the development of antiviral therapeutics. The HBV core antigen is a potential target and compounds affecting capsid assembly and/or function have been identified, but, to date, they have not been developed into therapeutic modalities [233–238].

Given the challenges with the development of antiviral therapeutics targeting viral proteins, an alternative approach is to target cellular gene products that are vital for the viral life cycle but are dispensable at some level for host viability. In this regard, our current understanding of the transcriptional regulation of HBV biosynthesis offers some cellular therapeutic targets that might potentially be exploited for the development of antiviral compounds. Nuclear receptors are ligand-dependent transcription factors governing the level of HBV 3.5 kb pregenomic RNA synthesis. Antagonists targeting HNF4α, PPARα, FXRα, or LRH1 could potentially lead to a reduction in HBV biosynthesis especially if viral transcription is reduced to a level where HBcAg dimers are expressed below the level required to support capsid assembly [200]. The limitations of nuclear receptors as antiviral targets include the functional redundancy resulting from multiple nuclear receptors governing HBV 3.5 kb pregenomic RNA synthesis and the possible undesirable effects on host metabolic function associated with their reduced activities, which might induce cellular toxicity. Targeting FoxA transcription factors at the appropriate developmental stage might be more challenging but potentially more therapeutically beneficial. Transient inhibition of FoxA activity during early neonatal development could potentially lead to the DNA methylation of viral genomes transmitted from mother to child at birth. This could lead to the transcriptional inactivation of the HBV cccDNA, which effectively and permanently terminates viral biosynthesis with the functional eradication of the viral replication intermediate that is refractory to current therapeutic modalities. The major challenge with this approach is the difficulty in effectively targeting FoxA while limiting any possible long-term negative effects on normal cellular and tissue physiology. Regardless of these challenges, the study of the transcriptional regulation of HBV biosynthesis has revealed several interesting aspects of both HBV and liver developmental biology while indicating a number of potential approaches to the development of novel therapeutic modalities targeting host gene products. Going forward, it is hoped that combinations of current and future therapies might result in effective treatments, leading to the resolution of chronic HBV infections and ultimately the worldwide eradication of this devastating human pathogen.

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