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Hong Tang *Editor*

Hepatitis B Virus Infection

Molecular Virology to Antiviral Drugs

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Preface

Hepatitis B virus (HBV) is a DNA virus, belonging to the *Hepadnaviridae* family. More than 50 years ago, the serendipitous discovery of "Australian antigen" as the HBsAg of HBV by Dr. Baruch S. Blumberg paved the way to the development of diagnosis, prevention, and treatment of HBV infection. HBV infection remains a global public health problem. The WHO estimates that globally, two billion people have been infected with HBV. In 2015, an estimated 257 million persons, or 3.5% of the world population, were living with chronic HBV infection. Uncontrolled chronic HBV infection can progress to life-threatening end-stage chronic liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC). In recent years, tremendous advances in the field of HBV basic and clinical research have been achieved. The deeper understanding of the key steps in the viral life cycle, the key regulators in the HBV transcription and replication, and the mechanisms of immunopathogenesis contribute greatly to the better clinical management of HBV infection. Current antiviral treatment with nucleos(t)ide analogs (NAs) or/and interferon enables full suppression of serum HBV DNA to undetectable levels in the majority of patients, thus leading to the improvement of liver function and the decreased incidence of cirrhosis and HCC. However, due to the difficulty in clearing the covalently closed circular DNA (cccDNA, the template for HBV transcription and replication), current anti-HBV agents and strategies can only achieve a clinical HBV cure in few patients. Thus, HBV infection remains a vital topic, and the advances in HBV basic and clinical research are crucial for achieving the ultimate goal of virological cure of HBV.

To review the achieved advances in the field of HBV study, we invite the world-leading experts in their respective fields to compile this book titled *Hepatitis B Virus Infection: From Molecular Virology to the Development of Antiviral Drugs*. This book aims to provide a state-of-the-art review of the current understanding of HBV genome and life cycle, the regulation of HBV transcription and replication, and the immunopathogenesis of HBV. It also provides an update of the cell and animal models employed in HBV basic and preclinical research. Those advances are the driving forces for the identification of new viral and host markers for guiding the clinical practice, as well as the identification of new therapeutic targets for the

development of novel antiviral agents. The last chapter of this book discusses the clinical management of chronic hepatitis B covering the standard and optimization antiviral therapies in treatment-naïve and treatment-experienced patients, as well as in the special populations. The up-to-date advances in the development of new anti-HBV drugs are also discussed. Future treatment option for achieving an HBV cure may be a combination of the current antiviral drugs and the newly developed antiviral agents targeting the different steps of the viral life cycle or the newly developed agents modulating the host immune responses.

I believe the content of this book provides a timely and comprehensive reference for medical students and young researches, as well as for experienced clinicians and researchers working in the field of HBV study. Hopefully, this book may also serve as a valuable source to enlighten the creative minds to develop new strategies or novel antiviral drugs to cure HBV infection in the foreseeable future. Finally, I would like to express my sincere appreciation to all the coauthors who provided great efforts and contributions to this book.

Chengdu, Sichuan, China Hong Tang

Contents

Chapter 1
Hepatitis B Virus Infection: Overview

Hong Li, Libo Yan, Ying Shi, Duoduo Lv, Jin Shang, Lang Bai, and Hong Tang

Abstract Hepatitis B virus (HBV) is a DNA virus, belonging to the *Hepadnaviridae* family. It is a partially double-stranded DNA virus with a small viral genome (3.2 kb). Chronic HBV infection remains a global public health problem. If left untreated, chronic HBV infection can progress to end-stage liver disease, such as liver cirrhosis and hepatocellular carcinoma (HCC). In recent years, tremendous advances in the field of HBV basic and clinical research have been achieved, ranging from the HBV biological characteristics, immunopathogenesis, and animal models to the development of new therapeutic strategies and new drugs against HBV. In this overview, we begin with a brief history of HBV discovery and treatment milestones. We then briefly summarize the HBV research advances, which will be detailed in the following chapters.

1 Discovery of HBV

As early as the 1950s, clinicians observed the hepatitis that occurred after blood transfusion and proposed the concept of serum hepatitis [1]. In 1964, Blumberg and Alter collected blood samples from all over the world for the study of lipoprotein polymorphisms and serendipitously observed an unusual reaction between serum from a transfused hemophilic patient and an Australian aborigine, and the new antigen was designated as the "Australian antigen" [2, 3]. By 1967, accumulating evidences from Blumberg's group and other research groups showed a strong correlation between the presence of the Australian antigen and serum hepatitis [4–8]. In 1970, David Dane used electron microscope to inspect the isolated virus particles from serum of patients with Australian antigen-associated hepatitis and demonstrated the famous "Dane particles" as the complete virus (42 nm in diameter) [9]. The outer

Hong Li and Libo Yan contributed equally to this chapter.

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surface protein of the Dane particle is the Australian antigen, which was later officially named by the World Health Organization (WHO) as the HBV surface antigen (HBsAg). In 1976, Blumberg was awarded the Nobel Prize in Physiology or Medicine for his scientific achievement in the discovery of HBV and his visionary concept in developing the first generation of plasma-derived HBsAg vaccine. In 1979, Galibert completed the whole genome sequence of HBV and demonstrated it as a partially double-stranded DNA virus with approximately 3200 base pairs of nucleotides in length [10], thus setting the stage for the second generation of HBV vaccine made from genetic engineering.

The development of HBV immunization began in 1971 by using HBV immunoglobulins to prevent HBV infection. In 1978, the efficacy of the first generation of HBV vaccine consisting of HBsAg particles made from the plasma of HBV carriers was successfully validated in clinical trials [11]. In 1979–1982, using modern molecular cloning methods, William Rutter cloned the HBsAg gene in *Escherichia coli* (*E. coli*) to express the HBsAg protein [12], thus opening the door for recombinant HBV vaccine [13]. Subsequently, the HBsAg gene was successfully transferred to yeast for the mass production of HBsAg as HBV vaccine [14]. In 1984, the yeast-expressed HBsAg vaccine prepared by Merck company was used to vaccinate chimpanzees, demonstrating that the vaccinated chimpanzees were totally protected from intravenous HBV challenge of human serum source [15]. Subsequently, the yeast-expressed HBsAg vaccine was approved by the US Food and Drug Administration (FDA) in 1986. In 1989, Dr. Roy Vagelos, on the behalf of Merck, provided all the technology needed to manufacture HBV vaccine to China with the largest burden of HBV infection. In 1991, HBV vaccine was incorporated into the neonatal immunization program by WHO, which significantly reduced the global HBV infection rate. Since 1992, HBV vaccination has been incorporated into the national routine immunization program by the Chinese government, which led to a markedly decline of HBV infection in China.

HBsAg is the most important marker for the diagnosis of HBV infection. Since the discovery of the "Australian antigen" by Blumberg, a variety of HBsAg qualitative detection methods have been developed, from the detection of HBsAg by immunoelectrophoresis in the 1960s to the enzyme-linked immunosorbent assay in the 1980s. Quantitative detection of HBsAg was introduced as early as in the 1990s, but fully automated and high-throughput quantitative assays have only been recently available. Serum HBsAg quantification not only serves as a useful test in clinical practice to define the specific immunological conditions of the single HBV carrier during the dynamic natural history of HBV infection but also a prediction marker of virological response to antiviral therapy and long-term prognosis [16, 17]. In addition to HBsAg, other commonly used HBV serum markers include anti-HBs, HBeAg and anti-HBe, and anti-HBc. HBV nucleic acid detection is mainly to detect HBV DNA level. HBV DNA testing has also undergone a process from qualitative to quantitative testing. The most widely used HBV DNA quantification method is the real-time polymerase chain reaction (PCR) method. Serum HBV DNA levels reflect the viral replication, and monitoring HBV DNA levels during treatment allows evaluation of antiviral therapy effect.

In the 1990s, interferon-α was approved by the US FDA for the treatment of HBV infection, marking the treatment of HBV infection into the era of antiviral therapy [18]. In the late 1990s, lamivudine became the first nucleos(t)ide analog (NA) approved for anti-HBV treatment, representing the treatment of HBV infection into the era of NAs [19]. At the beginning of the twenty-first century, the long-acting pegylated IFN (Peg-IFN) and the potent and low-resistant NAs entecavir (ETV) and tenofovir disoproxil fumarate (TDF) were approved for HBV treatment [18]. Long-term antiviral treatment with full suppression of serum HBV DNA to undetectable levels not only halts hepatic inflammation but also decreases the incidence of liver cirrhosis and HCC.

2 Epidemiology and Natural History of HBV Infection

HBV infection remains the most common chronic viral infection in the world. WHO estimates that globally, 2 billion people have been infected with HBV. In 2015, an estimated 257 million persons, or 3.5% of the world population, were living with chronic HBV infection [20]. About 887,000 people die each year from HBV-related liver disease with approximately half coming from China. In 2006, the epidemiology serosurvey of HBV in China revealed that the prevalence of HBsAg for population aged 1–59 years was 7.18% [21, 22]. Based on this calculation, there were about 93 million HBsAg carriers in China, of which approximately 20 million are chronic hepatitis B (CHB) patients [23]. The latest epidemiological data showed that the estimated national HBsAg prevalence in the general population was 6.1% in 2016 [24].

The natural history of HBV infection depends on the interaction among the virus replication and evolution, the host immune response, and the environment factors. The age at which HBV is infected is the most important factor affecting chronicity. The risks of progression from acute to chronic HBV infection are approximately 95% if the infection occurs during the perinatal period, 20%–30% in children aged 1–5 years, and less than 5% in adults. The natural history of chronic HBV infection can be characterized into four phases: (1) immune-tolerant phase, (2) HBeAg-positive immune-active phase, (3) inactive CHB phase, and (4) HBeAg-negative immune reactivation phase [25, 26]. In the immune-tolerant phase, the HBV-infected subjects are characterized by high levels of HBV DNA, positive HBeAg, but normal ALT levels and the absence of significant inflammation or fibrosis in the liver. HBV-infected subjects in this phase can be defined as HBV carriers. In the HBeAg-positive immune-active phase, the HBV-infected patients are characterized by elevated ALT and HBV DNA levels together with liver injury by liver histology, and therefore, those patients are defined as HBeAg-positive CHB patients [27]. The transition from the immune-active phase to the immune-inactive phase is reflected by the seroconversion from HBeAg to antibody to HBeAg (anti-HBe), the normal ALT levels, and the low or undetectable HBV DNA levels. However, around 10–30% of those patients who have HBeAg seroconversion continue to have

elevated ALT and high HBV DNA levels and, therefore, are defined as HBeAg-negative CHB patients [27]. Most of these patients have mutations in the pre-core or core promoter region. Patients with sustained liver injury and persistent HBV replication are prone to develop liver cirrhosis and HCC.

HBV infection not only leads to acute and chronic hepatitis but also is one of the most important etiological factors for liver cirrhosis and HCC. In the absence of antiviral treatment, the annual incidence of liver cirrhosis in CHB patients is 2–10% [28], the annual risk of progression from compensated to decompensated cirrhosis is 3–5%, and the 5-year survival rate in patients with decompensated cirrhosis is only 14%–35% [29]. The annual incidence of HCC in non-cirrhotic patients is about 0.5%–1.0% [29], whereas in cirrhotic patients, the annual incidence of HCC increases to 3–6% [28, 30, 31]. Liver cirrhosis has been classified by WHO as one of the top 10 causes of death in low-middle-income countries, and HCC as one of the top 10 causes of death in upper-middle-income countries [32].

3 Biological Characteristics and Immunopathogenesis of HBV

3.1 HBV Genome and Life Cycle

HBV particles are composed of the HBV genome, nucleocapsid, and the envelope proteins. The HBV genome is a partially double-stranded DNA with approximately 3200 base pairs. The longer-strand DNA is complementary to pregenomic RNA (pgRNA) and therefore is designated as minus (−) strand, whereas the shorter strand is designated as plus (+) strand. The (−) strand contains four overlapping open reading frames (ORFs) (PreC/C, P, PreS/S, and X). Under the joint regulation of the four promoters (the core promoter, the PreS1 promoter, the PreS2/S promoter, and the X promoter) and two enhancers (EnhI and EnhII), four distinct classes of HBV transcripts are transcribed: the 3.5 kb PreC/C mRNA, the 2.4 kb PreS1 mRNA, the 2.1 kb PreS2/S mRNA, and the 0.7 kb X mRNA. The PreC/C ORF is responsible for encoding HBeAg and HBcAg; the P ORF encodes the HBV DNA polymerase; the PreS/S ORF encodes the large (L), the middle (M), and the small (S) envelope proteins; and the X ORF is responsible for encoding the X protein (HBx).

The life cycle of HBV involves the viral entry into host cells; rcDNA's entry into the nucleus to form cccDNA; the expressions of viral RNAs and proteins; viral capsid assembly; reverse transcription and rcDNA formation; and, finally, viral packaging, maturation, and budding. Mediated by the antigenic loop (AGL) present in the S domain of HBsAg, HBV is initially attached to heparan sulfate proteoglycan (HSPG) on the surface of hepatocyte membrane [33]. Subsequently, through the preS1 region of the L protein, HBV is tightly bound to the sodium-taurocholate cotransporting polypeptide (NTCP) on the surface of hepatocytes [34, 35]. After HBV entry into the hepatocytes, the nucleocapsid is released, and the rcDNA of

HBV enters the nucleus to form cccDNA, which resides inside the hepatocyte as a microchromosome [36]. Current anti-HBV NAs have no direct effect on cccDNA, which explains why HBV infection is currently manageable but still incurable by current treatments. Under the action of host RNA polymerase II, cccDNA serves as the template for transcription of the abovementioned four HBV transcripts and the translation of seven viral proteins. Of note, in addition to encode the translation of HBcAg, HBeAg, and DNA polymerase, the 3.5 kb pgRNA also has an important function as a template for viral reverse transcription and replication. During viral replication, HBV DNA polymerase binds to the ε-stem-loop structure near the 5' end of the pgRNA, forming a specific pgRNA-polymerase ribonucleoprotein (RNP) complex, which is encapsulated by the core antigen polypeptide dimer to form an immature nucleocapsid [37]. Under the catalysis of HBV DNA polymerase in the nucleocapsid, the 3.5 kb pregenomic RNA serves as the template for reverse transcription of the (−) strand DNA, which subsequently serves as the template for the synthesis of the (+) strand DNA, thus forming the progeny rcDNA. The recycling of the de novo synthesized rcDNA into the nucleus makes more cccDNA, maintaining the cccDNA reservoir. Double-stranded linear (dsl) DNA may be generated due to erroneous viral DNA replication [38]. Mature nucleocapsid and envelope protein aggregate in the endoplasmic reticulum to complete the packaging, maturation, and viral budding [39]. The discovery of HSPG and NTCP and their crucial roles in HBV entry into hepatocytes and the immature nucleocapsid containing the pgRNA and the dslDNA has significantly advanced our understanding of the HBV life cycle.

3.2 HBV Transcription and Translation

Using the reporter gene system containing the HBV promoter, a series of *cis*-acting transcriptional regulatory sequence elements and *trans*-acting DNA-binding proteins have been discovered. The 3.5 kb pgRNA is primarily regulated by *cis*-acting regulatory sequence elements within the HBV genomic enhancer I (EnhI/Xp) and enhancer II (EnhII/Cp). HBV EnhI is located within the ORF P, between the ORF S and X, and overlaps with the X promoter, which enhances the transcription of C, SPI, SPII, and X promoters. The transcription factors reported bound to EnhI/Xp include C/EBP, P53, IRF, NF1, HNF3, HNF4, RXR, PPAR, COUPTF, RFX1, AP1, CREB, and ATF2 [40, 41]. EnhII is located to the upstream of Cp. The transcription factors bound to HBV EnhII/Cp include SP1, RFX1, C/EBP, FTF, HLF, E4BP4, HNF4, HNF3, RXR, PPAR, COUPTF1, and ARP1 [40, 41].

HNF4, RXR, PPAR, FXR, and LRH1 are liver-enriched transcription factors capable of supporting HBV replication in non-hepatoma cells [41, 42]. The identification of these liver-enriched transcription factors contributes significantly to the hepatocyte-specific tropism of HBV. In addition to the transcription factors, some transcriptional co-activators (including PGC1, CBP, SRC1, and PRMT1) and co-repressors (including SBP) are also involved in the regulation of HBV transcription

and replication [43–45]. In addition, other host proteins including APOBEC3B, PRMT5, and PRKAA/AMPK have also been reported to participate in the HBV transcription and replication [46–48].

The viral protein HBx can also act as a *trans*-acting factor possibly through interacting with certain host proteins to enhance HBV transcription and replication. It has been reported that HBx not only enhances HBV DNA replication but also activates the transcription of 3.5 kb HBV RNA. The augmentation of HBV transcription is always observed in parallel to HBV replication, strongly suggesting that the stimulation of HBV replication is mainly due to the enhanced transcription by HBx [49, 50]. It has also been suggested that HBx enhances HBV replication through posttranscriptional regulation mechanisms, such as the regulation of intracellular calcium signaling and activation of downstream Pyk2/FAK kinase [51]. Recent studies have found that HBx can promote the degradation of SMC5/6 to enhance HBV replication [52, 53].

3.3 Immunopathogenesis of HBV

The immunopathogenesis of CHB is complex and has not yet been fully elucidated. Numerous studies have shown that HBV is a non-cytopathic hepatotropic virus. Persistent liver inflammation plays an important role in the progression of CHB to cirrhosis and HCC. The innate immune response plays a role in the early stages of HBV infection and induces subsequent adaptive immune responses [54]. HBV inhibits the innate immune response by interfering with Toll-like receptors and retinoic acid receptors through the viral components such as HBeAg and HBx [55–59]. Although the innate immune response of CHB patients is impaired, antiviral cytokines of the innate immune pathway can still inhibit HBV. This is supported by the ability of IFNα or TLR agonists to induce innate immunity to inhibit HBV replication [60]. At present, many novel anti-HBV drugs are being developed to inhibit HBV replication through the upregulation of innate immunity.

Many CHB patients are characterized with impaired frequency and function of myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) in their peripheral blood. Immature mDC and pDC with decreased capacity to produce IFN-α are associated with tolerogenic T-cell responses and HBV persistence [61]. HBV-specific immune response plays a major role in HBV clearance. The virus-specific effector CD8+ T-cell response is central to HBV pathogenesis. Major histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T lymphocytes can induce hepatocyte apoptosis and secrete IFN-γ, thus inhibiting HBV replication through non-cytolytic mechanisms. In chronic HBV infection, apoptosis-prone HBV-specific CD8+ T cells, the reduced cytokine function and proliferative capacity, and the T-cell exhaustion contribute to HBV persistence [60].

4 Current Antiviral Drugs Against HBV and New Antiviral Drugs Under Development

4.1 Current Antiviral Drugs Against HBV

Current antiviral drugs for HBV include IFNs and NAs. The first milestone in CHB treatment is the utilization of IFN-α (IFN-α-2a, IFN-α-2b, and IFN-α-1b) produced by recombinant DNA technology. Meta-analysis has shown that conventional IFN-treated HBeAg-positive CHB patients have higher HBeAg seroconversion and HBsAg clearance, but lower cirrhosis and HCC incidence than the patients without IFN treatment [62, 63]. However, due to its limited efficacy, low sustained viral response, and frequent injections, the conventional IFN has been largely replaced with the long-acting Peg-IFN, which was approved by the US FDA for HBV treatment in 2002. International multicenter randomized controlled clinical trials showed that HBeAg-positive CHB patients treated with Peg-IFN-α-2a for 48 weeks resulted in HBeAg seroconversion rate of 32% at 24 weeks posttreatment follow-up [64] and the HBeAg seroconversion reached 41% at 48 weeks posttreatment follow-up [65]. Similar HBV DNA inhibition and HBeAg seroconversion can also be achieved in HBeAg-positive CHB patients treated with Peg-IFN-α-2b [66].

The second milestone in CHB treatment is the use of the lamivudine (LAM), which revolutionized the treatment of CHB. LAM exhibits good antiviral effects in HBeAg-positive and HBeAg-negative CHB patients, even in CHB patients with advanced liver diseases. However, resistance to LAM can be easily developed. It has been reported that LAM resistance can reach up to 80% after 5 years of treatment [67]. Following LAM, adefovir dipivoxil (ADV) was the second antiviral drug approved for anti-HBV therapy. However, like LAM, ADV has low genetic barrier and drug resistance can be easily developed and another drawback for ADV is its nephrotoxicity [18]. Telbivudine (LdT) is another nucleoside analog for antiviral treatment of CHB. With a proven safety profile, LdT is a pregnancy category B medication and has been applied to prevent mother-to-child-transmission (MTCT) in mothers with HBV infection [68–70]. However, similar with LAM and ADV, long-term LdT treatment leads to high rate of drug resistance (34% after 3-year TBV therapy) [71].

The third milestone in CHB treatment is the clinical use of the potent and low-resistant NAs: ETV and TDF. Both ETV and TDF can strongly inhibit HBV replication and have high genetic barrier to drug resistance. It has been shown that the 3-year cumulative ETV resistance rate is 1.7–3.3% [72]. Resistance to TDF was not detected in CHB patients after 6 years of TDF monotherapy [73]. Most CHB patients with long-term use of ETV or TDF can achieve histological improvement and even the reversal of liver fibrosis. Tenofovir alafenamide (TAF) has been recently approved for treatment of CHB in adults. Compared to TDF, TAF has a better safety profile (lower rates of bone and renal abnormalities) and similar

antiviral efficacy. Therefore, current international guidelines recommend the use of Peg-IFN, ETV, TDF, and TAF as first-line therapeutic options for CHB, while the NAs with low genetic barriers (LAM, ADV, and LdT) are no longer recommended as the first-line antiviral agents in treatment-naïve CHB patients.

4.2 Optimization Treatment Strategies Based on Current Antiviral Drugs

Currently, most CHB patients treated with NAs or Peg-IFN monotherapy can achieve sustained viral suppression, whereas the difficulty in achieving HBsAg loss or the elimination of cccDNA remains the major obstacle for the cure of CHB. Theoretically, the combination of NAs and Peg-IFN may have a synergistic therapeutic effect to enable more CHB patients achieving HBsAg loss. Many clinical studies have been conducted to investigate the efficacies of different optimization strategies of NAs and Peg-IFN combination [64, 74–83].

One of the combination strategies is the simultaneous administration of NAs and Peg-IFN (the de novo combination). However, the initial de novo combination of LAM plus Peg-IFN and ADV plus Peg-IFN showed less-than-desirable results in treatment-naïve patients [64, 74, 75]. Recently, the de novo combination of TDF and Peg-IFN in treatment-naive CHB patients for 48 weeks led to increased rate of HBsAg loss at week 72 (9.1%) than those receiving Peg-IFN (2.8%) or TDF (0%) alone [78]. However, a recent randomized controlled, open-label study did not support the advantage of de novo combination of NA and Peg-IFN in CHB patients [77].

The other strategy to combine NA and Peg-IFN is the sequential combination, which means the "add-on" or "switch-to" strategy to CHB patients who are already on NA treatment. The "early add-on" strategy was investigated in the ARES study by comparing 24 weeks of ETV followed by 24 weeks of Peg-IFN add-on versus 48 weeks of ETV monotherapy for treatment-naïve HBeAg-positive CHB patients [79, 80]. The results showed no favorable effect of the combination strategy. The "late add-on" strategy was investigated in the PEGAN study enrolling only HBeAg-negative CHB patients with undetected HBV DNA by at least 1 year of NA treatment [81]. In the PEGAN study, patients were randomized to either continue NA or add on Peg-IFN treatment for 48 weeks. The results showed that HBsAg loss rates were significantly higher in the full-dose Peg-IFN add-on group than in the NA group [81].

The "early switch-to" strategy was investigated in the OSST study enrolling HBeAg-positive patients who had received 9 to 36 months of ETV therapy with HBeAg <100 PEIU/ml and HBV DNA ≤ 1000 copies/ml [82]. The enrolled patients in the OSST study were randomized to receive ETV or switch to Peg-IFN-α2a for 48 weeks. The "late switch-to" strategy was investigated in the New Switch study enrolling HBeAg-positive patients who achieved HBeAg loss and HBV DNA < 200 IU/mL with previous NA treatment (ADV, LAM or ETV). The patients were randomized to receive Peg-IFN for 48 or 96 weeks [83]. Both the OSST study

and the New Switch study demonstrated a significant increase of HBsAg loss in the Peg-IFN switch group than the NA monotherapy group, and CHB patients with low baseline HBsAg level and on-treatment HBsAg response are more likely to benefit from the "switch-to" combination therapy [82, 83].

4.3 Advances in the Development of New Anti-HBV Drugs

The development of new anti-HBV drugs can be summarized into two categories: [1] new direct-acting antiviral drugs targeting the different steps of HBV life cycle, and [2] new indirect antiviral drugs modulating host immune response to inhibit or potentially eradicate HBV. The direct-acting antiviral drugs under development include HBV entry inhibitors, the therapeutic approaches targeting HBV cccDNA, RNA interference (RNAi)-based agents, capsid assembly inhibitors/modulators, new NAs targeting HBV polymerase, ribonuclease H (RNaseH) inhibitors, and HBsAg release inhibitors. The indirect antiviral drugs to suppress HBV via modulating the host innate or adaptive immunity include TLR-7 and TLR-8 agonists, retinoic acid-inducible gene 1 (RIG-1)/nucleotide-binding oligomerization domain protein 2 (NOD-2) agonists, programmed death receptor 1 (PD-1) inhibitors, and different kinds of therapeutic vaccines.

4.3.1 Direct Antiviral Drugs Against the Life Cycle of HBV

HBV entry inhibitor Myrcludex-B targeting the NTCP receptor is currently under phase II clinical trial. It has been shown that Myrcludex-B not only inhibits HBV DNA replication but also reduces cccDNA formation [84, 85]. Genome-editing technologies including transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system, which can be designed to target HBV cccDNA sequences, represent highly promising therapeutic tools to achieve the ultimate goal of curing CHB [86–91]. However, specific and efficient delivery of the gene editing sequences to HBV-infected hepatocytes and the risk of the intrinsic off-target effects of the genome-editing technologies are big challenges that need to be met in the future. The next-generation RNAi agents that target HBV transcripts to reduce viral antigen, HBV DNA, and cccDNA levels are also being developed. For example, the RNAi-based agent ARC-520 is currently in phase II clinical trial [92]. The nucleocapsid assembly inhibitors including NVR 3–778 [93–95], JNJ-6379 [96], GLS4 [97], and ABI-H0731 [98] are currently under different phases of clinical trials. Several new NAs currently under different phases of clinical trials include Besifovir (LB80380/BSV, a new acyclic nucleotide analog) [90, 99–101] and CMX157 (phase II) [102, 103]. The RNaseH inhibitors are also promising candidates for developing new anti-HBV drugs [104, 105]. The HBsAg release inhibitors include REP2139 or its analog REP2165 which have progressed to phase II clinical trials. The results

showed that the combination of REP2139-Mg or REP2165-Mg (250 mg iv qW) with TDF and Peg-IFN led to HBsAg loss or seroconversion in a high proportion of CHB patients (http://replicor.com/, as of August 2019).

4.3.2 Indirect Antiviral Drugs that Modulate Host Immune Response to Control CHB

Indirect antiviral drugs that exhibit anti-HBV effect may function through modulating the host innate or adaptive immune response. The rationale behind the development of indirect antiviral drugs to modulate the host innate immunity is that the HBV-infected hepatocytes have impaired innate immune response; thus reactivating the host innate immune response may lead to the control of HBV infection. For example, Toll-like receptor 7 (TLR-7) and TLR-8 agonists are currently under clinical trials [106, 107]. In chronic HBV infection, T-cell immune tolerance and the T-cell exhaustion contribute to HBV persistence. Sustained high expression of the programmed cell death-1 (PD-1) in T cells plays an important role in T-cell exhaustion; thus, blocking the PD-1 pathway using the anti-PD-1 antibody (nivolumab) could be a major immunotherapeutic strategy to treat HBV infection [108, 109]. In addition, the therapeutic vaccines to treat HBV infection are also being developed [110–114].

In summary, since the discovery of HBV by Blumberg and Alter in the 1960s, tremendous advances in the field of HBV basic research, prevention, and clinical control of HBV infection have been achieved. A deeper understanding of the HBV life cycle and HBV immunopathogenesis, together with the development of cell culture models and animal models for HBV study, will further drive the development and testing of new therapeutic agents against HBV infection. Future treatment options for HBV cure may be a combination of multiple antiviral drugs, either the combination of different direct antiviral drugs targeting the various steps of HBV life cycle or the combination of direct antiviral drugs with host immune modulators. With the optimization treatment strategies based on current antiviral drugs and the newly developed antiviral agents, the ultimate cure of HBV infection will be achieved in the foreseeable future.

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Chapter 2
HBV Genome and Life Cycle

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Abstract Chronic hepatitis B virus (HBV) infection remains to be a serious threat to public health and is associated with many liver diseases including chronic hepatitis B (CHB), liver cirrhosis, and hepatocellular carcinoma. Although nucleos(t)ide analogues (NA) and pegylated interferon-α (Peg-IFNα) have been confirmed to be efficient in inhibiting HBV replication, it is difficult to eradicate HBV and achieve the clinical cure of CHB. Therefore, long-term therapy has been recommended to CHB treatment under the current antiviral therapy. In this context, the new antiviral therapy targeting one or multiple critical steps of viral life cycle may be an alternative approach in future. In the last decade, the functional receptor [sodium-taurocholate cotransporting polypeptide (NTCP)] of HBV entry into hepatocytes has been discovered, and the immature nucleocapsids containing the non- or partially reverse-transcribed pregenomic RNA, the nucleocapsids containing double-strand linear DNA (dslDNA), and the empty particles devoid of any HBV nucleic acid have been found to be released into circulation, which have supplemented the life cycle of HBV. The understanding of HBV life cycle may offer a new instruction for searching the potential antiviral targets, and the new viral markers used to monitor the efficacy of antiviral therapy for CHB patients in the future.

1 Introduction

Hepatitis B virus (HBV) belongs to *Hepadnaviridae* family and is the causative factor of chronic hepatitis B (CHB). Worldwide, 257 million people are chronically infected with HBV, and 887,000 people annually die of HBV infection-related end-stage liver disease, such as liver cirrhosis, liver failure, and liver cancer [1–3].

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Currently, there are two kinds of antiviral agents used for CHB treatment, including nucleos(t)ide analogues (NA) and pegylated interferon (Peg-IFN-α), and neither of them can directly target and efficiently clear the covalently closed circular DNA (cccDNA) which persists in the nuclei of the infected hepatocytes [4, 5]. Since cccDNA is the main cause that CHB is difficult to cure, the eradication of cccDNA is an ideal goal for the cure of CHB. Unfortunately, it is almost impossible via the currently available antiviral therapies. Alternatively, the functional cure, a state characterized with serum HBsAg loss, has been suggested. Since functional cure is also difficult to achieve, the long-term NA therapy has been recommended in almost all the guidelines for CHB management [6–9]. However, the long-term therapy may bring a series of problems, such as side effects, costs, and compliance.

Recent discoveries allow a better understanding of HBV life cycle and pave the way for identifying the multiple new therapeutic targets for CHB, as well as provide the new viral markers for guiding the clinical practice. Firstly, the HBV entry inhibitors are being developed after the discovery of NTCP as a major functional receptor of HBV infection [10–12]. Meanwhile, the approaches specifically targeting cccDNA are being explored in experimental models when the gene editing technologies discovered, such as the systems of zinc-finger nucleases, TAL effector nucleases, and CRISPR-associated (cas) nucleases [13–20]. Moreover, other antiviral agents for CHB treatment are being developed, such as the uses of RNA interference to inhibit HBV replication, capsid assembly modulators (CAMs) to inhibit pgRNA encapsidation and block HBV DNA synthesis, and immune modulatory therapies [21–29]. In the future, the combination of the current and the newly developed antiviral agents targeting the different steps of viral life cycle may be an alternative approach for achieving the eradication of HBV infection and the clinical cure of CHB. Besides, several serum viral markers, including hepatitis B core-related antigen (HBcrAg), HBV RNA, and dslDNA, are going to be the potential viral markers for monitoring the efficacy and prognosis of antiviral therapy for CHB patients [30–39].

2 HBV Genome

HBV has a genome of approximately 3.2 kb and partially double-stranded, relaxed circular DNA (rcDNA) which is composed of a complete coding minus strand (−) and an incomplete noncoding plus strand (+) with a fixed 5' end and a variable-size 3′ end [40, 41]. As shown in Fig. 2.1, the relaxed circular configuration of HBV genome is maintained by the cohesive end regions containing two direct repeats (DRs) of 11 nucleotides (TTCACCTCTGC) termed DR1 (nt 1824–1834) and DR2 (nt 1590–1600) [42]. Both DR1 and DR2 play the important roles in viral replication, and the integration of HBV DNA sequences into host cell genome [43]. The coding minus strand contains four overlapping open reading frames (ORFs) (preC/C, P, preS/S, and X), four promoters [core promoter (CP, 1613-1849) consists of the upper regulatory region (URR, nt1613-1742) and the basic core promoter

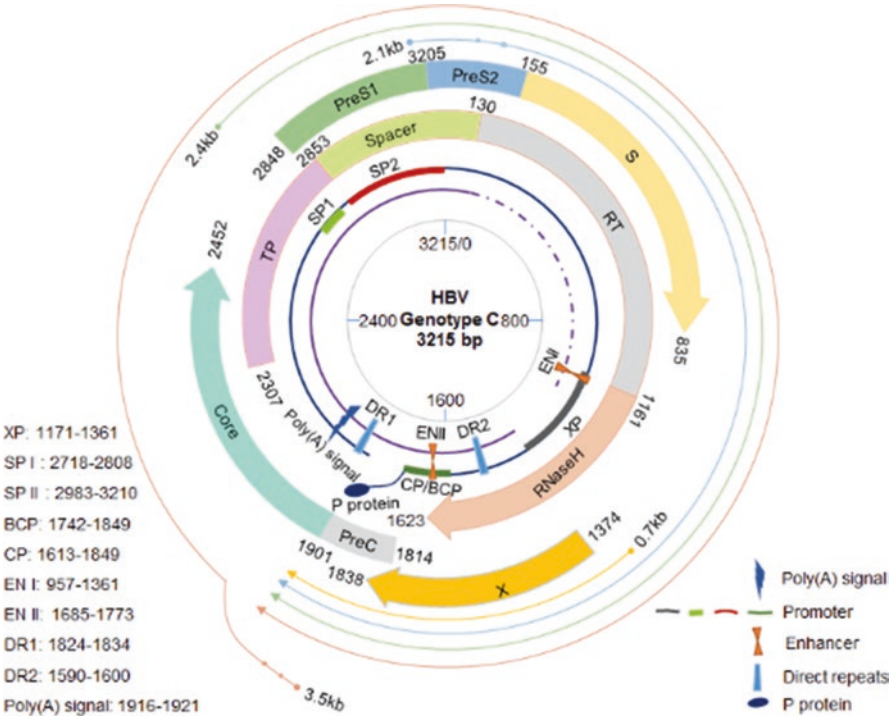

Fig. 2.1 The circular diagram of HBV genome. The locations of ORFs and important regulatory elements refer to genotype C HBV genome [45, 46]. XP, X promoter; SP I, PreS1 promoter; SP II, PreS2 promoter; BCP, Basic core promoter; CP, Core promoter; EN I, Enhancer I; EN II, Enhancer II; DR1, Direct repeat 1; DR2, Direct repeat 2; Poly(A), Polyadenylation

(BCP, nt 1742-1849), PreS1 promoter (SP I, nt 2718-2808), PreS2 promoter (SP II, nt 2983-3210), and X promoter (XP, nt 1171-1361)], and two enhancers [Enhancer I (EN I, nt 957-1361) and Enhancer II (EN II, nt 1685-1773)], as well as polyadenylation [poly(A)] signal (nt 1916-1921) [44, 45]. Under the regulation of four promoters and two enhancers, the 3.5, 2.4, 2.1, and 0.7 kb polyadenylated HBV RNAs are generated, respectively [46, 47].

The preC/C ORF and P ORF overlap each other partially: the former encodes hepatitis B e antigen (HBeAg) and core protein, and the latter is responsible for the synthesis of HBV DNA polymerase (P protein). Core protein and P protein are translated from pregenomic RNA (pgRNA), while HBeAg is translated from precore mRNA. Both pgRNA and precore mRNA are transcriptionally regulated by CP in which the URR regulates the promoter activity and the BCP regulates the transcriptions of both pgRNA and precore mRNA [48, 49]. Core protein self-assembles to form the viral capsid and binds with cccDNA to participate in its epigenetic modifications [50], while P protein consists of four pivotal domains including terminal protein (TP) domain, spacer region, reverse transcriptase (RT) domain, and

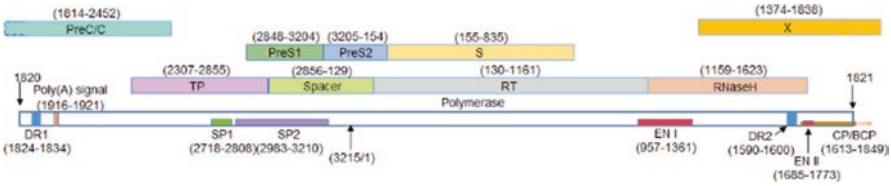

Fig. 2.2 The linear diagram of HBV genome

RNase H domain, which is anchored on the 5′ terminus of the minus-strand DNA and has many functions in the viral life cycle, such as viral RNA binding, pgRNA encapsidation, protein priming, reverse transcriptase activity, DNA polymerase activity, and RNase H activity [51–54]. The precursor of HBeAg undergoes proteolytic processing in the endoplasmic reticulum (ER) and generates the mature HBeAg [55]. Although HBeAg is not essential for the viral replication and infection, such a secretory protein has immune regulatory functions. For example, HBeAg can inhibit host innate immunity and mediate immune evasion by inducing T cell tolerance [56, 57]. The preS/S ORF is located within P ORF and uses three different in-frame AUG start codons to encode three envelope glycoproteins including large (L), middle (M), and small (S) surface antigens (HBsAg). L-HBsAg is translated from the 2.4 kb HBV RNA transcriptionally regulated by SP I, and the latter two envelope glycoproteins are translated from 2.1 kb HBV RNA transcriptionally regulated by SP II [58]. The X ORF encoding X protein (HBx) is the smallest ORF and overlaps with P ORF. HBx is translated from the 0.7 kb HBV RNA which is transcriptionally regulated by XP (Figs. 2.1 and 2.2).

In addition to the promoter elements, the expressions of these viral genes are also modulated by two enhancer elements, EN I and EN II. EN I is located between ORF S and X and is consisted of a 5′ modulatory element, a central core domain with actual enhancer activity and a 3′ domain that overlaps with X ORF [59, 60]. EN II is located at the upstream of CP and partially overlaps with BCP and URR, which comprises region IIA and IIB potent enhancer elements [47, 48]. Both EN I and EN II have the ability to upregulate the activities of the HBV promoters in an orientation-independent manner, in which EN I preferentially upregulates the activities of CP/BCP and XP but has a modest effect on the activities of SP I and SP II, while EN II preferentially upregulates the activities of the SP I, SP II, and XP [48, 49, 61, 62].

3 Viral Entry

HBV is highly species-specific and hepatotropic. This species specificity is partly dependent on the expression pattern of HBV entry receptors. As shown in Fig. 2.3, HBV has been identified to firstly attach to heparan sulfate proteoglycans (HSPGs) on hepatocyte membrane with low affinity, which is mediated by the antigenic loop (AGL) present in the S domain of all HBsAg [63]. Subsequently, PreS1 region of

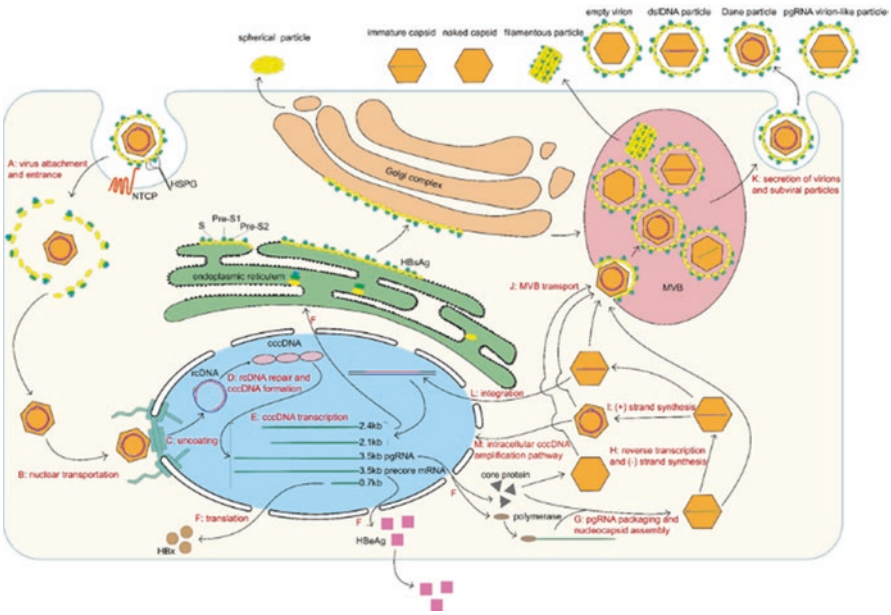

Fig. 2.3 HBV life cycle. (**a**): The entrance of HBV Dane particles into hepatocyte via the binding of L-HBsAg to NTCP and binding of S-HBsAg to HSPG on the membrane of hepatocyte. (**b**): The release of nucleocapsid and the transportation of nucleocapsid into nucleus where the uncoating takes place. (**c**): The release of rcDNA from the nucleocapsid into nucleus of hepatocyte. (**d**): The conversion of rcDNA to cccDNA which serves as the template for viral transcription. (**e**): The transcriptions of cccDNA to HBV RNAs. (**f**): The translations of HBV RNAs result in the productions of HBeAg, core protein, P protein, L-HBsAg, M-HBsAg, S-HBsAg, and HBx protein. (**g**):The binding of P protein to ε region of pgRNA and the recruitment of core proteins to assemble nucleocapsid. (**h**): The reverse transcription and the synthesis of HBV minus (−)-strand DNA using pgRNA as the template. (**i**): The accurate translocation of P protein and the synthesis of HBV plus (+)-strand DNA. (**j**): The envelopment of the nucleocapsid via MVB transport pathway. (**k**): Secretion of virions and subviral particles. (**l**): Incorrect translocation of P protein resulting in formation of dslDNA and the integration of dslDNA into the host genome. (**m**): Nuclear translocation and uncoating of newly formed nucleocapsids to replenish cccDNA pool

L-HBsAg, predominantly the 2-48 N-terminal amino acids (aa), binds to NTCP on the basolateral membrane of hepatocytes with high affinity [64]. The myristoylation of the N-terminal PreS1 facilitates virus infection through enhancing the capability of receptor recognition [65–67]. NTCP composed of 349 aa is a conjugated bile acid transporter, and the aa 84-87 and 157-165 of NTCP are critical for viral entry into hepatocytes [10, 68]. Next, the viruses are internalized through endocytosis, including the caveolae- and clathrin-mediated endocytosis [69–71]. The subsequent endosomes are translocated by a common vesicle traffic pathway relying on cytoskeleton and are regulated by Rab, small guanosine triphosphatases of the Ras superfamily, to deliver the endosomes to different cellular compartments [72, 73].

During this process, the translocation of vesicle is accompanied by a pH decrease from about 6.2 in early endosomes to approximate 5.5 in late endosomes, resulting in the fusion of the viral envelope with the endosomal membrane to release nucleocapsids [74]. However, pH-independent entry and sequential endosomal sorting seems to be the major determinants in the infection of duck hepatitis B virus (DHBV) [75]. Besides, the cholesterol on viral membrane is required for the above endosomal escape of the virus into the cytosol [76]. The nucleocapsids are transported by motor proteins along microtubules toward the nucleus [77]. The nucleocapsids are directed to nucleus pore complex (NPC) by nuclear localization sequence (NLS) of core proteins and then are arrested at the nuclear basket by Nup153, a component of NPC [78, 79]. Finally, the nucleocapsids disassemble, followed by HBV rcDNA and some core proteins releasing into nucleus [80].

4 The Conversion of rcDNA to cccDNA

Once rcDNA enters the nucleus, it will go through a series of biochemical steps to be converted to covalently closed circular DNA (cccDNA), which is the crucial intermediate that serves as the template of HBV replication during HBV life cycle (Fig. 2.3) [81]. Many host factors, such as DNA repair devices, participate in the conversion of rcDNA to cccDNA [82, 83]. Firstly, P protein anchored on the 5′ terminus of the minus-strand HBV DNA may be removed by human tyrosyl DNA phosphodiesterase-2 (TDP2) which is a host repair enzyme that can remove topoisomerase (TOP)-DNA covalent complexes [84–87]. Except for TDP2, this deproteinization reaction may also be achieved by an endonucleases-mediated nucleolytic pathway [87]. Meanwhile, a capped and 18 nucleotide-long RNA oligomer at the 5′ end of plus-strand DNA and the 9 or 10 nucleotide-long terminally redundant segment (r sequence) at the 5′ end of minus-strand DNA are removed by flap structure-specific endonuclease 1 (FEN1) which specifically cleaves the 5′-flap structure formed by RNA oligomer or r sequence [88–90]. However, other host factors other than FEN1 may also participate in removing the flap structure in rcDNA [90]. Next, the plus-strand DNA which is variable in length is extended not only by P protein but also host cellular polymerases, such as DNA polymerase κ (POLK) [88, 91, 92]. POLK is a key host cellular polymerase supporting HBV infection, while other host DNA polymerases, such as DNA polymerase L (POLL) and H (POLH), also participate in this step [92]. Finally, to converse rcDNA to cccDNA, both linear strands are mainly ligated by host cellular DNA ligase (LIG) 1 and 3 after the extension of plus-strand DNA [93]. Besides, LIG4 has been considered to participate in the formation of cccDNA from double-strand linear DNA (dslDNA) through the nonhomologous end joining (NHEJ) DNA repair pathway [93–95].

Once formed, cccDNA will be organized into a chromatin-like viral minichromosome and modified by host histone proteins, such as H3 and H4 histone proteins, as well as nonhistone proteins like viral HBx, core protein, and host epigenetics-related proteins, whereas the nucleosome spacing (repeat length) is 180 bp which is

different from the 200 bp repeat length for the chromatin of eukaryotic cells [96, 97]. HBV cccDNA can be epigenetically modified to regulate viral replication and viral gene expression, including DNA methylation and histone modifications [98].

5 The Expressions of Viral RNAs and Proteins

After rcDNA is being converted to cccDNA in the nucleus of hepatocyte, cccDNA is used as the template of HBV replication and then transcribed into the 3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb HBV RNAs [46, 81, 99–101]. As shown in Fig. 2.3, there are two kinds of the 3.5 kb HBV RNAs, the precore mRNA and pgRNA. The precore mRNA is mainly transcribed from 1783 nt of HBV genome and is longer than pgRNA which mainly transcribed from 1818 nt [102]. HBV RNAs share the same 3′ end terminus using a classic poly(A) signal "UAUAAA" (nt 1916-1921) (Fig. 2.1) [36, 38, 46]. However, there is another cryptic poly(A) signal "CAUAAA" within the X ORF (nt 1788-1793), which can lead to the productions of the truncated HBV RNAs (trRNA) [38, 103].

As mentioned above, the transcriptions of HBV RNAs are regulated by four promoters (CP, SP I, SP II, and XP) and two enhancers (EN I and EN II) [101, 104]. CP is consist of BCP and URR. BCP contains four serial TATA-like box, in which the three ahead are used to control the transcription of precore mRNA and the fourth one is used for controlling the transcription of pgRNA [105, 106]. URR is consist of a positive regulatory element (nt 1613-1636) and a negative regulatory element (1636–1742), both of which regulate the transcriptional activity of BCP [107–109]. SP I initiates the transcription of 2.4 kb mRNA, and SP II initiates the transcription of 2.1 kb mRNA [110]. XP initiates the transcription of 0.7 kb HBV RNA [111]. EN I promotes the transcriptions of precore mRNA, pgRNA and 0.7 kb HBV RNAs, but has a modest effect on the transcriptions of the 2.4 kb and 2.1 kb HBV RNAs, while EN II preferentially promotes the transcriptions of the 2.4 kb, 2.1 kb, and 0.7 kb HBV RNAs [48, 49].

HBV pgRNA can be spliced by the formation of spliceosome which could remove introns like the cellular machineries [112, 113]. Just like the intron of heterogeneous nuclear RNA (hnRNA) which is composed a 5′ donor site ("GU"), a 3′ acceptor site ("AG"), a branch site (usually "A" base), and a polypyrimidine tract [113], the deleted HBV pgRNA sequences are also mainly shown a GU-AG manner [114]. The most abundant HBV pgRNA splicing variant is termed as SP1, with nearly one third of the HBV genome deleted (from nt 2447 to 489) [112]. There are also other forms of spliced pgRNA utilizing the different 5′ donor site and 3′ acceptor site [112]. Interestingly, the spliced pgRNAs can also be encapsidated and subsequently reversed transcribed [115–118]. Moreover, SP1 can be translated into HBV splicing-generated protein (HBSP) which is reported to influence cell viability, proliferation, and migration, as well as the TNF-α signaling pathway [119–123].

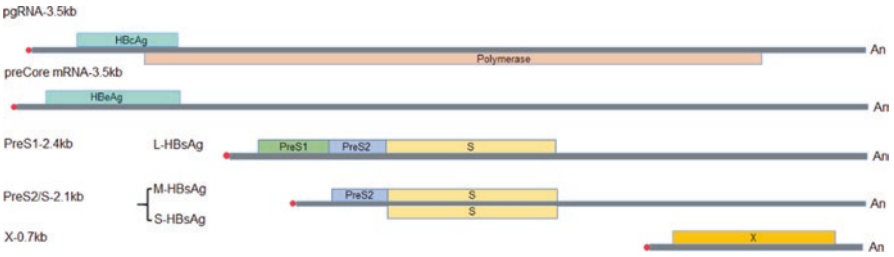

Fig. 2.4 The linear diagram of HBV RNAs and viral proteins

Subsequently, HBV RNAs are translocated into cytoplasm [99]. As shown in Fig. 2.4, the precore mRNA is firstly translated to precore polypeptide, of which the first 19 amino acids in the N-terminal region is a signal peptide trafficking precore polypeptide to the ER where the signal peptide and the 34 amino acids of C-terminal domain are removed to form the mature HBeAg [57, 124]. The mature HBeAg can be released directly into circulation [46]. Meanwhile, a part of pgRNAs are translated to core protein and P protein, and the other parts of pgRNAs are encapsulated into the nucleocapsid and serve as the templates for viral replication. The 2.4 kb HBV RNA is translated to L-HBsAg, and the 2.1 kb HBV RNA is translated to M- and S-HBsAg. Once L-, M-, and S-HBsAg are synthesized at ribosome, they will be sorted into the ER for processing and then be transferred to the Golgi apparatus for further processing. Subsequently, these HBsAg can form two kinds of subviral particles [125, 126]. The predominant subviral particles are spherical particles with a diameter of approximately 20 nm, which mainly contain S-HBsAg and are secreted via the Golgi pathway of host cells [125, 127, 128]. The other subviral particles are less numerous filamentous particles (almost 1% of the spherical particles) with a diameter of approximately 22 nm, which contain a majority of S-HBsAg proteins and equal amounts of M- and L-HBsAg [125, 127–131]. Unlike spherical particles, filamentous particles are secreted by the host cell endosomal sorting complexes required for transport (ESCRT) and the multivesicular bodies (MVB) pathway [132]. Besides, the 0.7 kb HBV RNA is translated to HBx [99, 133, 134]. HBx is a multifunctional protein that is known to activate viral and host gene transcriptions, affect DNA repair processes, as well as regulate cell growth and death [135, 136].

6 Viral Capsid Assembly, Reverse Transcription, and rcDNA Formation

P protein recognizes the epsilon (ε) stem-loop including a bulge and an apical loop near the 5′ end of pgRNA, which is the encapsidation signal of pgRNA. A P-ε ribonucleoprotein (RNP) complex is formed by structural alterations of both pgRNA and P protein [137–140]. Meanwhile, chaperones and ATP may assist the RNP complex to be the right conformation for the subsequent encapsidation, and the

priming reaction occurs at this stage [141–143]. The RNP complex is recognized and encapsidated by core protein dimers to form core particles before or after the priming reaction, and the subsequent reverse transcription occurs inside the core particles [140]. However, there may be some other packaging signal-like RNA motifs termed as preferred site (PS) for core protein binding in pgRNA, which are found by RNA SELEX assays and share a purine-rich loop recognition motif-RGAG (R = purine) [144].

The hydroxyl group of tyrosine (Tyr) in the TP domain of P protein covalently binds with the first deoxyribonucleotide in the bulge region of ε stem-loop near the 5′ end of pgRNA to initiate the reverse transcription (Fig. 2.5a) [140, 145–148]. Next, the first four (TGAA) or three nucleotides (GAA) of the nascent minus-strand DNA are originated from the bulge region of ε stem-loop, followed by translocating the oligomer covalently linked to the P protein from ε stem-loop to the DR1 at the 3′ terminus of pgRNA and leading to the elongation of minus-strand DNA (Fig. 2.5b) [149–151]. Except for the Tyr residue in TP domain, the YMDD motif in RT domain of P protein are also required for the priming activity and are important to covalently link the first deoxyribonucleotide [146–148].

It is proposed that the primer-P protein complex is arranged through a *cis*-acting element termed Phi (φ) and located between DR2 and 3′ DR1 which is complementary

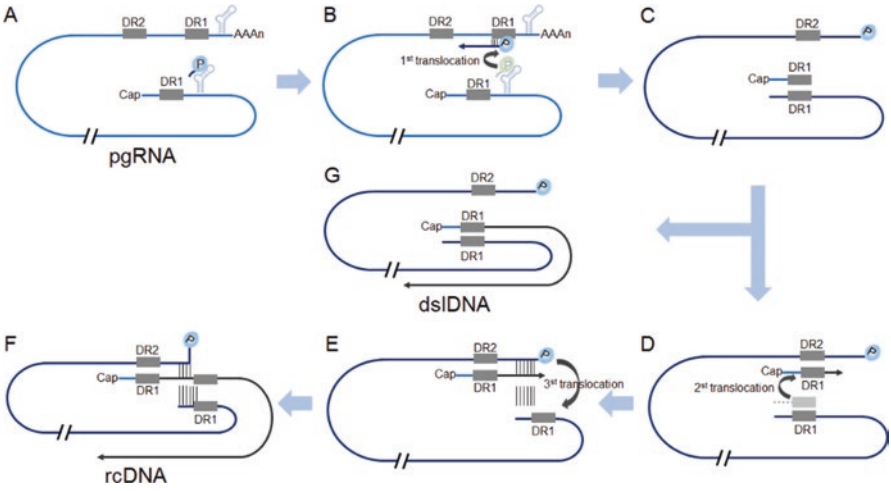

Fig. 2.5 The diagram for the formations of rcDNA and dslDNA from pgRNA. (**a**): HBV P protein-mediated priming at the ε region near the 5′ end of the pgRNA to initiate the reverse transcription process. (**b**): The first template translocation of the nascent DNA primer from ε to DR1 near the 3′ end of pgRNA. (**c**): The synthesis of minus-strand DNA and pgRNA digestion mediated by RNaseH domain of P protein. (**d**): The second template translocation of the pgRNA primer from DR1 to DR2 in the synthesis of plus-strand DNA. (**e**): The synthesis of plus-strand DNA toward 5′ end of minus-strand DNA and the third template translocation of the nascent plus-strand DNA from the 5′ end to the 3′ end of minus-strand DNA. (**f**): The formation of rcDNA in progeny virus with partial plus-strand DNA. (**g**): The formation of dslDNA through in situ priming of plus-strand DNA at the 3′ end of minus-strand DNA

to the half of 5′ ε stem-loop, thus the 5′ ε stem-loop and 3’ DRI are held in close proximity [152, 153]. In addition, another *cis*-acting element termed omega (ω) locates at the downstream of 3′ DR1 and can anneal with φ, which is also thought to be important for minus-strand DNA synthesis [154]. Following the first translocation or switch, P protein extends the minus-strand to the 5′ end of pgRNA. During the minus-strand elongation, the RNase H domain of P protein degrades pgRNA concomitantly from the pgRNA-DNA complex, whereas an oligoribonucleotide (16-18 ribonucleotides) of 5′ terminal pgRNA is reserved when the synthesis of minus-strand DNA terminated at the 5′ end of pgRNA (Fig. 2.5c) [155–157]. Notably, since the location of DR1 is within the large terminal redundancy of pgRNA, the de novo synthesized 3′ terminal minus-strand also has a terminal redundancy termed r sequence, which plays an important role in the plus-strand DNA synthesis [158].

The oligoribonucleotide of the 5′ end capped-pgRNA serves as the primer for plus-strand DNA synthesis [156, 159]. The capped-RNA oligomer (RNA primer) encompassing the DR1 sequence translocates (second switch) to the complementary DR2 sequences at the 5′ end of minus-strand and starts the synthesis of plus-strand (Fig. 2.5d) [156, 159, 160]. Once the plus-strand extends to the 5′ end of minus-strand DNA, the third translocation or switch from the 5′ end to the 3 ′ end of the minus-strand DNA will take place to continue the plus-strand DNA synthesis and form rcDNA (Fig. 2.5e and f) [54, 160]. Alternatively, without the successful second translocation, the RNA primer may remain at the 3′ end of minus-strand DNA and carry out the in situ priming of plus-DNA to form dslDNA (Fig. 2.5g) [54]. The dslDNA is preferred to be integrated into the host genome and then serves as the transcription template for HBsAg, whereas its production is a minor pathway with an occurring frequency of about 5%-20% under the normal conditions [161, 162]. Besides, several studies suggest that other *cis*-acting sequences in the minus-strand DNA may also participate to help spatially juxtapose through base pairing for plus-strand DNA synthesis [163, 164].

7 Viral Budding

The rc- or dslDNA containing core particles are termed as nucleocapsids. Some of nucleocapsids shuttle back to the nucleus to maintain a relatively stable pool of cccDNA, and other nucleocapsids are enveloped by HBV envelope glycoproteins (Fig. 2.3) [165–167]. For the latter process, two cytoplasmic domains (matrix domains, MDs) of Golgi-processed HBsAg in the MVB membrane contact with nucleocapsids, and such contacts will order the envelope proteins into a tightly packed formation in the MVB membrane and subsequently drive the inward budding process [125]. Since MD1 is located at the boundary between preS1 and preS2 in L-HBsAg (aa 103 to 124), and MD2 is located at the C-terminal half of the cytoplasmic loop between transmembrane domain (TM) 1 and 2 in S-HBsAg, it is

indicated that both L- and S-HBsAg are necessary for the inward budding while M-HBsAg is not essential [168–172]. Finally, the inward-budded nucleocapsids are sorted into the ESCRT complexes of host cell to catalyze the membrane fission and subsequently release outside the cell [131].

As shown in Fig. 2.3, except for the mature nucleocapsids, the immature nucleocapsids containing the non- or partially reverse transcribed pgRNA may also be enveloped and secreted in a similar way to the mature nucleocapsids [34, 35, 173, 174]. Besides, the empty capsids, referring to the capsids devoid of any form of HBV nucleic acid due to the core dimers failing to package HBV pgRNA, can also be enveloped by contacting with MD2 of S-HBsAg and released outside the cell as empty virions. Unlike the mature nucleocapsids, core proteins in empty capsids are mostly phosphorylated and may be the aberrant core proteins [126, 144, 175, 176].

In addition, it has been reported that the naked capsids can also be directly released outside the cell, which may be depended on the interaction between the HBV core particles and the Bro1 domain of Alix which act as a regulator of capsid releasing but independent of the ESCRT machinery (Fig. 2.3). However, the detail mechanism of this pathway has not been elucidated yet [177–179].

8 Conclusion

With an enveloped 3.2 kb rcDNA genome, HBV belongs to *Hepadnaviridae* family [180]. HBV particles enter hepatocytes through a high-affinity binding of the myristoylated viral preS1 to NTCP and a low-affinity binding of S-HBsAg to HSPG [10, 127, 181]. Subsequently, rcDNA enters into the nucleus and is converted to cccDNA, which persists as a minichromosome to transcribe HBV RNAs through the cellular transcription machinery. Among HBV RNAs, pgRNA is reverse transcribed to form HBV minus-strand DNA and encodes core protein and P protein. The assembly of viral capsid is initiated by binding of P protein to pgRNA, and then the encapsidated pgRNA is reverse transcribed to minus-strand DNA, followed by incompletely synthesizing the plus-strand DNA to form rcDNA [182]. The synthesized rcDNAs can either re-enter the nucleus to replenish cccDNA pool or be enveloped and released as viral particles [165, 183]. Except for rcDNA, dslDNA is generated when failing to translocate the RNA primer, which is often integrated into the host genome using the host enzymes [184, 185]. The integrated viral DNA fragments are frequently ended at the DR-1/2 regions of HBV genome [186, 187]. Since the intact ORF of S gene is present in the integrated viral DNA fragments, HBsAg can also be expressed from the integrated HBV DNA fragments [127, 188, 189]. Accordingly, HBsAg can be produced by either cccDNA or the integrated HBV DNA, and it has been reported that HBsAg may majorly originate from the integrated HBV DNA in HBeAg-negative HBV-infected individuals [127, 190, 191].

Like the nucleocapsids containing rcDNA, the nucleocapsids containing RNA, and the nucleocapsids containing dslDNA, the immature nucleocapsids and the empty particles may also be enveloped and released by ESCRT-dependent pathway

in MVB, which provides supplement to the traditional HBV life cycle (Fig. 2.3). These new discoveries of HBV life cycle may provide the new viral markers used for predicting the efficacy of antiviral therapy and offer the instructions for developing the new antiviral approaches.

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Chapter 3
The Regulation of HBV Transcription and Replication

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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.

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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

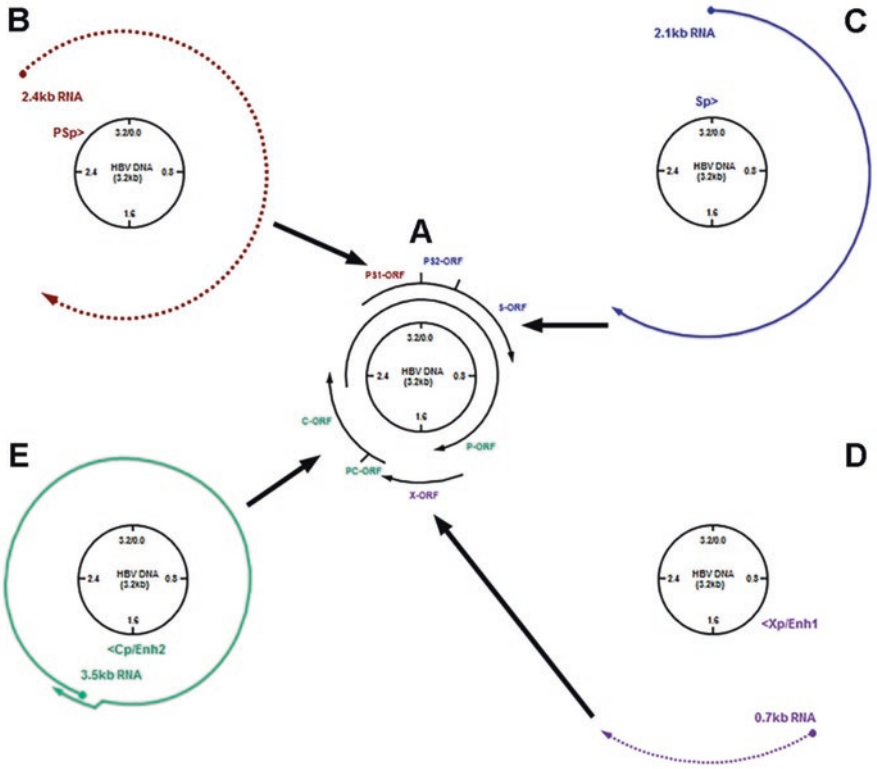

Fig. 3.1 Organization of the HBV genome. The circular HBV genome (subtype ayw) is 3182 nucleotides in length. The position of nucleotide coordinates 800 (0.8), 1600 (1.6), 2400 (2.4), and 3182 (3.2/0.0) are indicated. (*A*) The viral open reading frames (ORFs) are represented by black arrows. Orientation 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

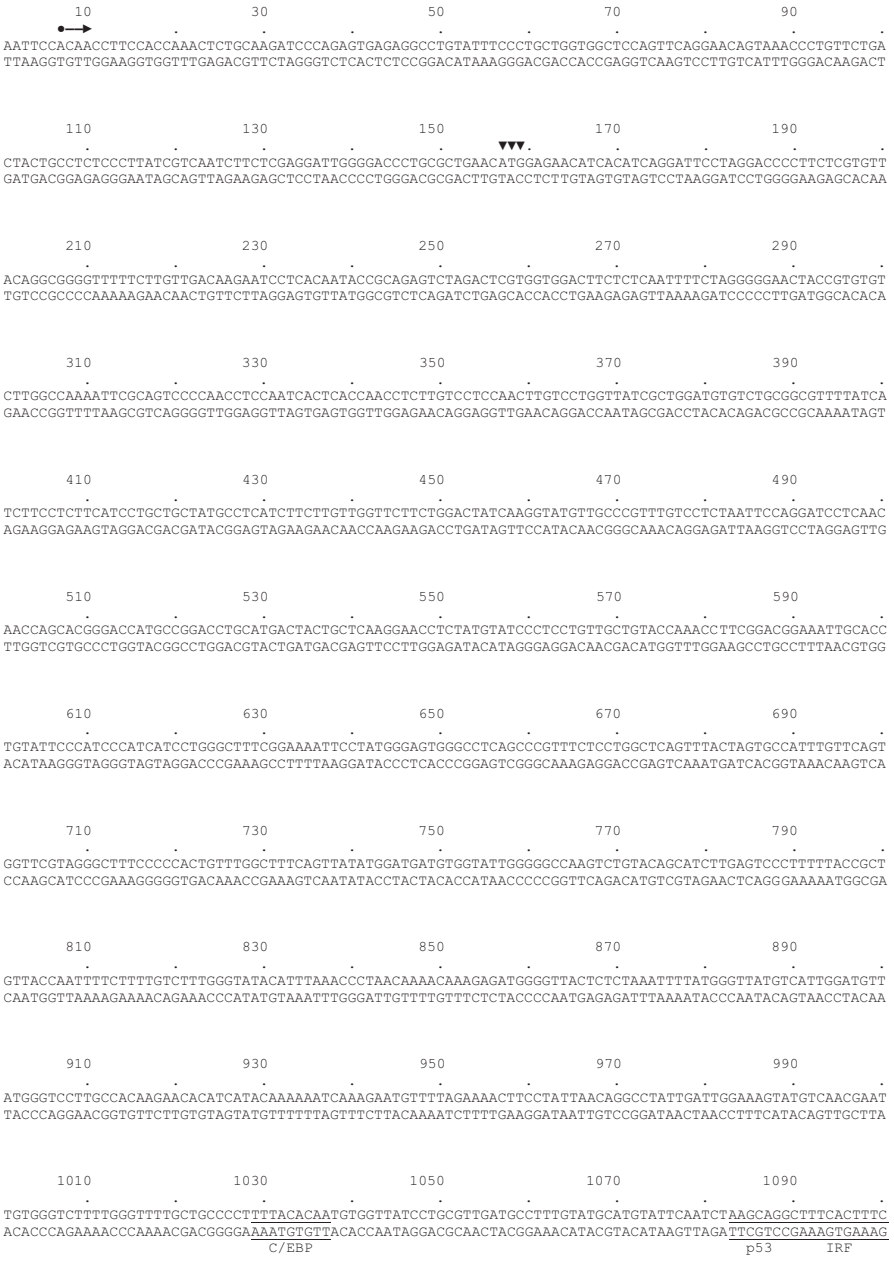

Fig. 3.2 Nucleotide sequence of the HBV genomic DNA (subtype *ayw*) showing the location of the transcription factors binding to the enhancer 1/X-gene promoter, enhancer 2/core promoter region, the intragenic core gene sequence, the large surface antigen promoter, and the major surface antigen promoter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

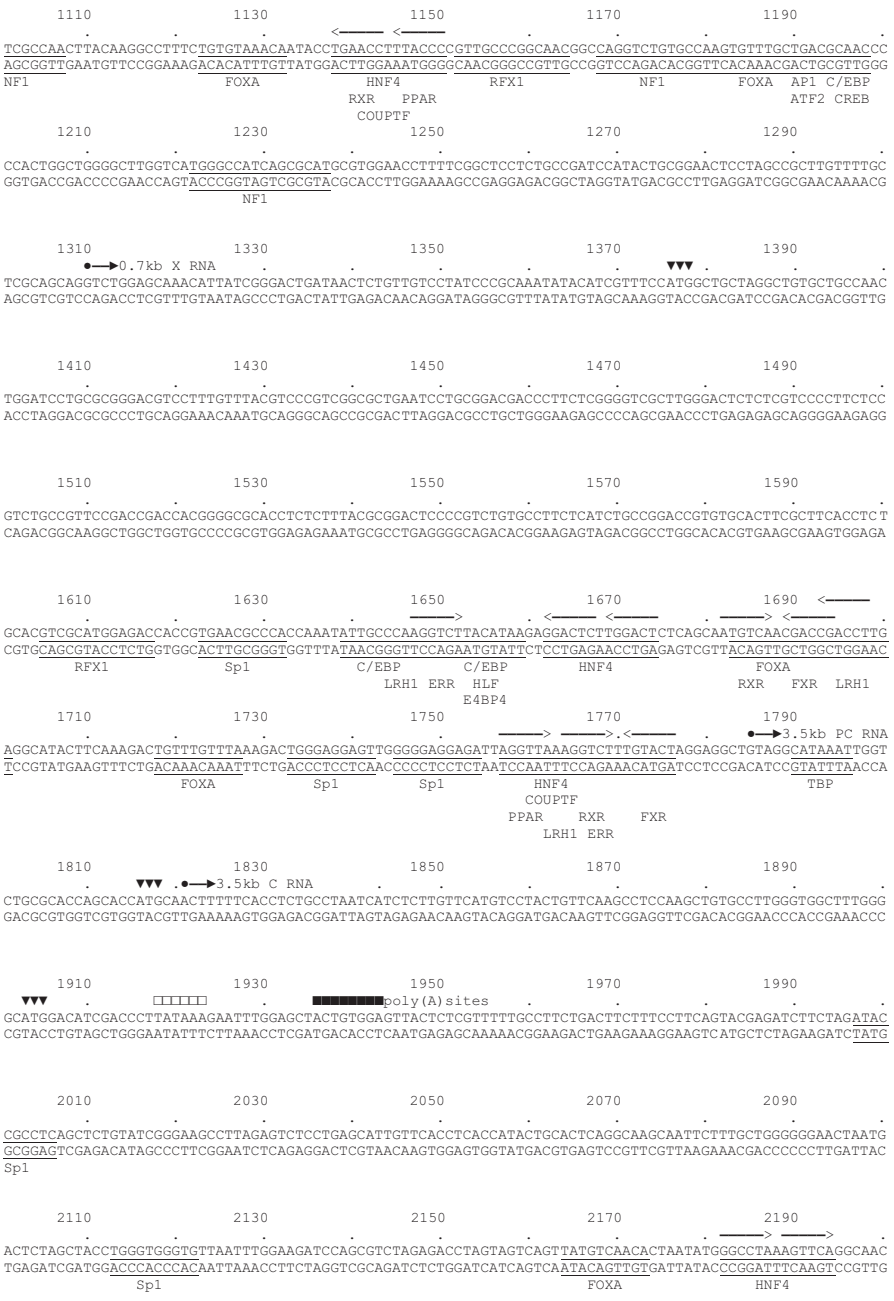

Fig. 3.2 (continued)

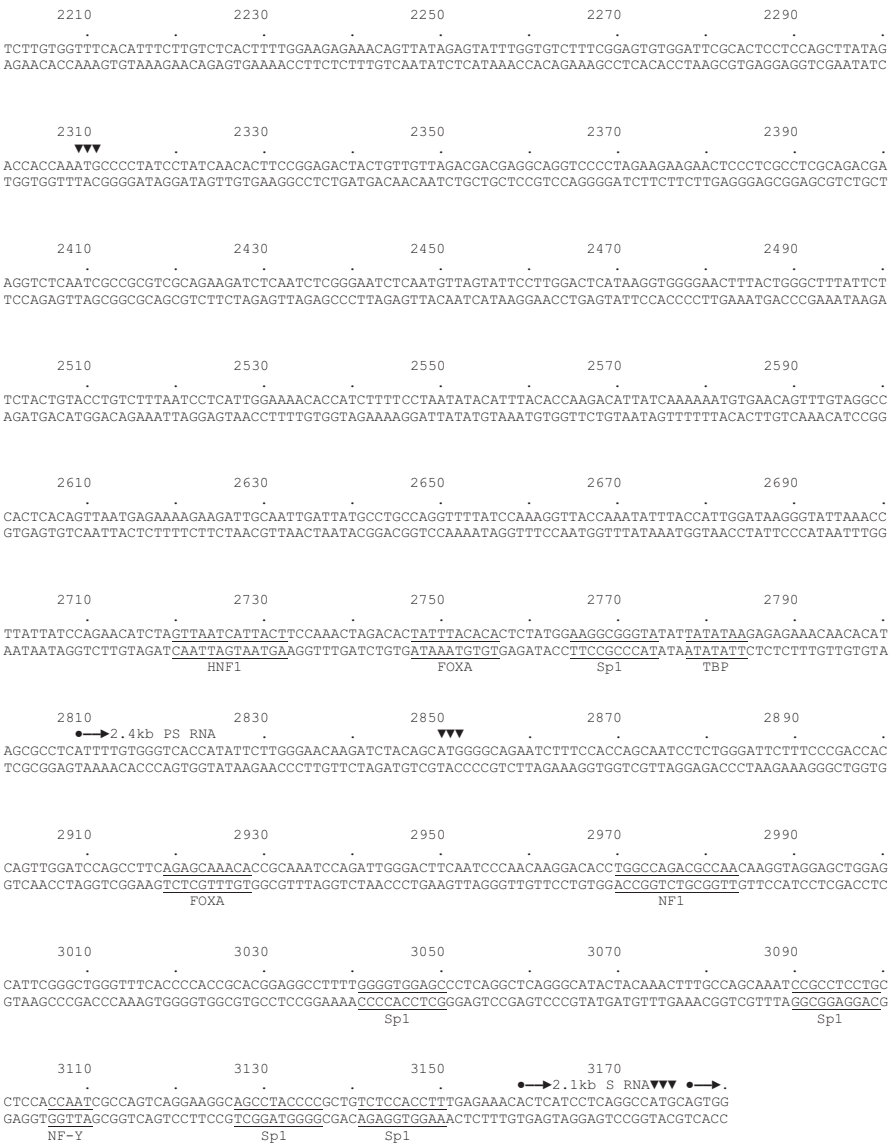

Fig. 3.2 (continued)

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

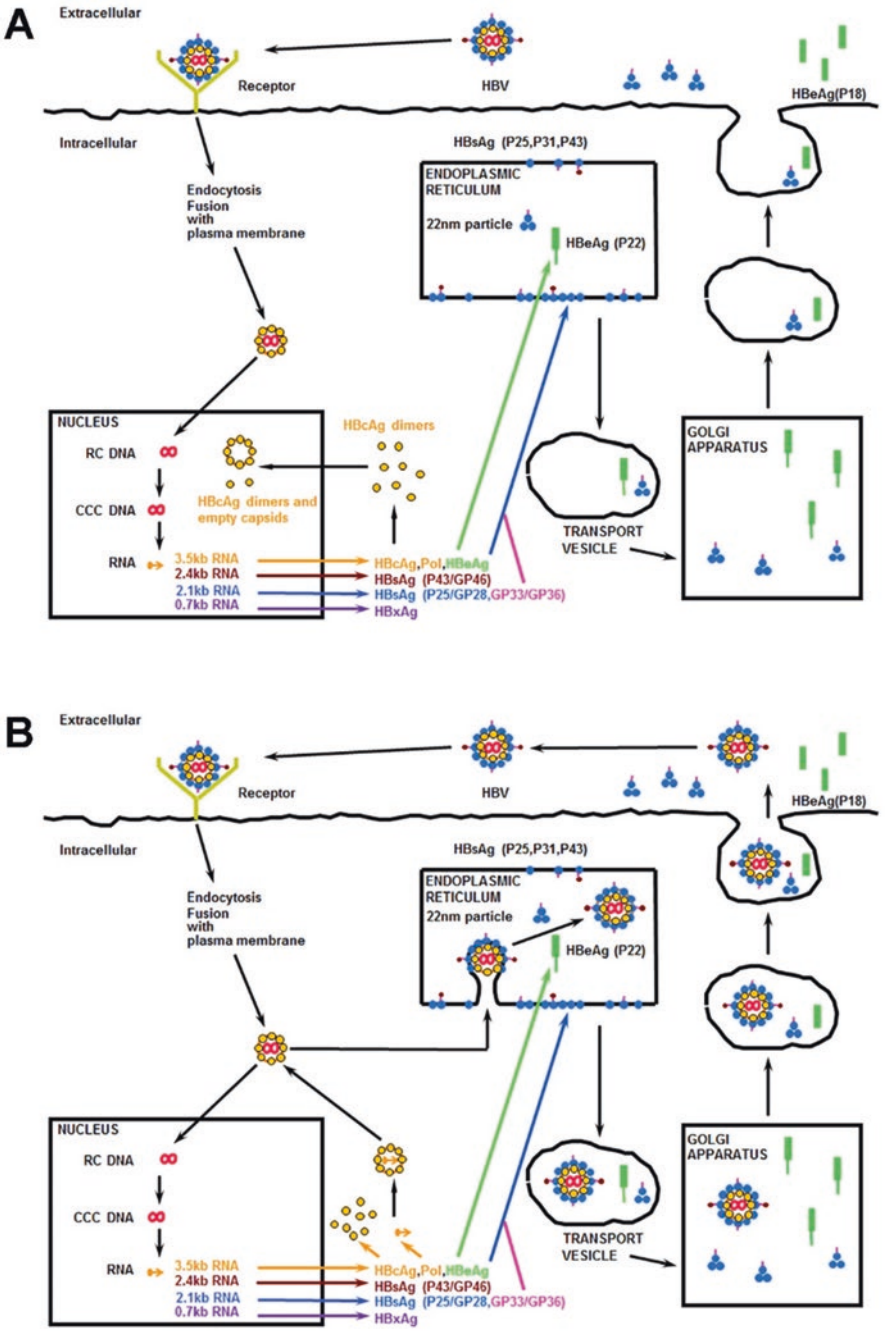

Fig. 3.3 The HBV replication cycle showing the intracellular pathway for the synthesis and secretion of HBV, HBsAg, and HBeAg polypeptides. (**a**) Lower levels of the HBV pregenomic 3.5 kb RNA preclude cytoplasmic dimer oligomerization, immature capsid formation, and hence HBV DNA synthesis. (**b**) 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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Chapter 4
Immunopathogenesis of HBV Infection

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Abstract More than 95% of adult infected with HBV show acute self-limited infection and eventually eliminate the virus. In contrast, about 90% of people exposed to HBV in early childhood develop chronic infection. The specificity of the virus and the host's antiviral immune responses together determine the outcome of HBV infection. It is generally believed that viral genome variation, viral titers, and inhibition of viral components against the host immune system are associated with persistent infection and liver damage. The dysfunction of innate immune cells (NK cells, monocyte/macrophages, NKT cells, etc.) and adaptive immune cells (antigen-presenting cells, T cells, B cells) is a key factor leading to virus clearance failure and liver inflammation. In this chapter, we summarize these viral factors and host factors in acute and chronic hepatitis B and update recent understanding of the immune-tolerant phase and pathological mechanisms associated with age and vertical transmission. This will help us to understand more fully the mechanisms of chronic HBV infection and liver injury and to develop combined treatment strategies of direct antiviral drugs for HBV life cycle and immunomodulators.

1 Introduction

Chronic hepatitis B virus (HBV) infection is one of the important causes of hepatitis, liver cirrhosis, and hepatocellular carcinoma. About 650,000 people worldwide die every year from various end-stage liver diseases caused by HBV infection. Although current preventive vaccines against HBV have proven to be safe, effective, and widely used, there is still a lack of thorough cure for the vast majority of people with chronic HBV infection. The outcome of HBV infection depends

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primarily on the interaction of the virus with the host's immune system. HBV replication itself does not cause liver cell damage in a short period of time, but the host's antiviral immune response against HBV can cause liver inflammation and hepatocytes damage while eliminating the virus, thus participating in the pathogenesis of HBV. The process has an important impact on the clinical progression and outcome of hepatitis B. The host's immune response against HBV mainly includes two parts: innate immunity and adaptive immunity. Among them, the main innate immune responses are induced by pattern recognition receptor (PRR), natural killer cells (NK cells), NKT cells, and monocytes/macrophages; the main adaptive immune responses include $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes. An effective innate immune response not only exerts a direct antiviral effect but also has an important impact on the HBV-specific immune response. HBV-specific T lymphocytes and B lymphocytes responses are decisive factors in the clearance of free virus and virus-infected hepatocytes and play a long-term immune surveillance function for HBV. In the process of chronic HBV infection, the virus evades and inhibits the antiviral effects of the innate immune and adaptive immune system through various mechanisms, thereby achieving its continuous replication, and the host also exhibits dysfunction of various immune cells.

2 Viral Factors Leading to HBV Persistence

2.1 HBV Genetic Variants

Biological characteristics of HBV usually cause the error-prone HBV polymerase, thus creating the viral genetic variability. Analyses on certain HBV mutants proved that there is a close association with mutants and unique clinical manifestations, which may affect the natural course of the infection and confer resistance to antiviral agents. The best characterized mutants related to viral persistence are the pre-core (pre-C) stop codon mutations and HBV surface (HBsAg) gene mutants.

The pre-C/C region variation was correlated with HBeAg-negative CHB and hepatitis B disease progression [1, 2] and also affected the response of antiviral drugs therapy [3, 4]. Recent studies have demonstrated an immunomodulatory role of HBeAg in antigen presentation and recognition by CD4+ T cells [5]. Patients with HBeAg-negative CHB are more likely to progress to severe hepatitis, cirrhosis, and hepatocellular carcinoma [6–8].

Mutations have been also identified in regulatory genetic elements of HBV genome. HBV core promotor mutations clustered in enhancer II, which are predominantly found in patients with a more aggressive course of disease such as fulminant [7] or chronic hepatitis B (CHB) and some of them are accompanied with a decrease or loss of HBeAg [9]. The biological phenotype of enhanced viral

replication is a common hallmark of core promoter mutations, which has been verified in transfected hepatoma cell lines [10] and primary hepatocytes [11]. Since not all fulminant hepatitis (HF) patients exhibit a high-replication phenotype [12], further studies are needed to address the additional mechanisms for the pathogenesis of FH.

Another mutant is HBsAg mutant. This mutant exhibited a defect in the S region of HBV genome to arginine at amino acid position 145 with loss of the group-specific antigenic determinant a [3], which is the main target of the vaccine response. This viral mutant was able to escape the immune surveillance and thereby resulted in an infection despite the presence of anti-HBs antibodies [13]; also, it resulted in impaired S secretion and decreased virion stability [14]. In patients after liver transplantation for HBV-related chronic liver disease who had received monoclonal or polyclonal anti-HBs antibodies to prevent reinfection of the graft [15] and in anti-HBs-positive individuals, the existence of HBsAg mutant resulted in immune escape [16].That's why the mutant is identified with "immune escape mutants." The emergence of the variant may potentially contribute to occult HBsAg-negative HBV infection.

2.2 Impact of Viral Load on HBV Persistence

The ability of HBV to establish persistence is also affected by the size of the viral inoculum, which was demonstrated in a chimpanzee infection model [17]. With a low-dose inoculum of 10^0 or 10^1, genome-equivalent (GE) of HBV would lead to massive spread of the virus to 100% of hepatocytes and viral persistence; animals inoculated with a high-dose inoculum of HBV showed limited spread of the virus to hepatocytes followed by abrupt viral clearance. The HBV clearance in chimpanzees is associated with a synchronized CD4 and CD8+ T cell response to HBV. The depletion of CD4+ T cells before and during a limited infection does lead to HBV persistence. It also coincided with a sharply synchronized influx of HBV-specific CD8+ T cells into the liver [17].

Previous studies indicated that, when viral load was high, interferon (IFN)-α/β suppressed HBV replication via transcriptional and post-transcriptional regulations. However, when viral load was low, IFN-α/β treatment induced HBV persistence in HBV transgenic mice or hydrodynamic injection mouse model [18, 19].The enhancement of HBV gene expression and replication was the result of stimulating the activity of enhancer I in HBV genome via interacting STAT3 with hepatocyte nuclear factor 3γ (HNF3γ). This observation indicated that HBV might actually use the host IFN response to stimulate its own replication and spread in the early stage of viral infection.

3 Host Genetics

Genome-wide association studies (GWAS) have proven to be very useful in discovering the host genetic factors. HLA genes encode the molecules that present antigens to T lymphocytes, and polymorphisms in these genes may alter the specificity and strength of antigen binding, affecting the T cell-mediated immune responses [20]. HLA-DP (rs3077 and rs9277535) [21] and HLA-DQ (rs7453920 and rs2856718) [22] SNPs were found to be susceptibility loci for HBV persistence.

EHMT2 is a histone lysine methyltransferase involved in gene expression regulation participating in immune cell development and differentiation, and TCF19 is a cell survival and proliferation-dependent transcription factor. An investigation in Koreans revealed that two new novel loci, marked by rs652888 in EHMT2 and rs1419881 in TCF19, had independent effects on HBV persistence [15]. However, probably due to the differences in genetic structures, the associations of rs652888 (EHMT2) and rs1419881 (TCF19) were not replicated in Chinese [23] and Thai [24] populations. Comparing chronic HBV carriers in Han Chinese with HBV naturally cleared individuals showed that rs3130542, located near HLA-C within the HLA region, accounted for HBV persistence [25].

UBE2L3 encodes a ubiquitin conjugating enzyme, which enhances NF-κB activation upon CD40 stimulation in B cells and tumor necrosis factor (TNF) stimulation in monocytes [26]. GWAS revealed that SNP rs4821116, located in UBE2L3 on chromosome 22, is the protective variant [27]. Another newly found locus was rs7000921, which was an eQTL (expression quantitative trait loci) for the nearby INTS10 gene [23]. Some analysis showed that INTS10 was involved in suppressing HBV replication in hepatocyte cell lines, likely through an IFN-dependent mechanism [20]. So, it's a protective allele associated with elevated INTS10 expression in theliver [28].

4 Innate Immune Responses

4.1 HBV Recognition by Innate Sensors

The innate immune system is the most primitive aspect of the host defense mechanism. Innate immune activation is facilitated through PRRs that recognize pathogen-associated molecular patterns (PAMPs). Among PRR, there are toll-like (TLR), retinoic acid-inducible gene I (RIG)-like, nucleotide-binding oligomerization domain-containing protein (NOD)-like, C-type lectin, and DNA-sensing receptors, which are differentially or ubiquitously expressed in various types of professional and nonprofessional immune cells [29].

Whether HBV is genuinely detected by PRR upon very short exposure or after the amplification of its genome and expression of its proteins during its replicative life cycle in hepatocytes is controversial. Some studies showed that HBV exposed

to a number of PRRs forms the first line of defense against infections. Surveys such as that conducted by Arik have shown that HBV capsid is a ligand for TLR2 [30] and ligand-activated TLR2 leads to the strong production of pro-inflammatory cytokines [30–32]. It has also been suggested that HBsAg activates DC via sCD14-dependent mechanism [33], which is the ligand lipopolysaccharide (LPS) coreceptor that facilitates activation of TLR4 [34], subsequently enhancing the capacity to activate antigen-specific cytotoxic T cells (CTL). The RIG-I has been shown to have a dual antiviral effect on HBV pgRNA. RIG-I binding to ε-stem loop of the HBV pregenomic RNA induces a strong induction of type III IFN [35]. Also, it counteracts the interaction of HBV polymerase with the ε-" region, suppressing viral replication [36]. It was also recently demonstrated that the cytosolic DNA sensor, cGAS, can recognize HBV DNA, leading the suppression of IRF3 expression and supporting HBV persistence in an infected cell [37].

In contrast, the view suggests that PRR-mediated innate immune responses are not activated early in HBV infection. No natural immune response was detected in the liver of HBV-infected chimpanzees [38]. Moreover, in the 3D microfluidic primary human hepatocyte (PHH) culture system, no IFN response was detected in both PHH and Kupffer cells (KCs) infected with HBV [39]. The reason for the difference between the two conclusions is that in vitro models used to study HBV recognition by innate sensors are suboptimal. Monolayer hepatocytes do not mimic the path of HBV actually entering the liver; and there is no standard procedure for HBV inoculum, such as HBcAg synthesized in bacteria may be contaminated by lipopolysaccharide-like ligands (TLR4 ligands) [40]. In addition, it has not been clearly demonstrated that HBV virion purification from patients can be used to in vitro/in vitro reproducible and effective infection of hepatocytes [41].

Taken together, the researches to date haven't been able to demonstrate the molecular determinants of a potential recognition of HBV PAMPs by PRRs. This understanding is instrumental for the development of PRR agonists, which could be used in immune-therapeutic combinations.

4.2 HBV-Driven Inhibition of Innate-Signaling Pathways

HBV was thought to be a stealth virus [42] as previous studies of patients with acute HBV infection [38] indicated that its infection doesn't lead to a strong activation of IFN and pro-inflammatory responses. Similar to this observation, a demonstration came from a microarray study performed with the biopsies of HBV experimentally infected chimpanzees; no specific induction of IFN-stimulated genes (ISGs) was detected [43]. In agreement with these findings, in an experiment using uPA-SCID mice grafted with human hepatocytes, only a slight increase of intrahepatic ISGs was detected after HBV infection [44].

HBV or viral proteins have been suggested to interfere with innate signaling pathways and thus attenuate the anti-HBV intrinsic immune responses [45]. HBsAg and HBeAg could inhibit the expression of TLR2-mediated IL-12 and TNF-α

production in monocytes/macrophages by interfering with JNK activation [46, 47]. Moreover, the production of cytokines in response to both TLR2 and TLR4 ligands by PBMCs from CHB patients was inhibited, and it was related to the level of HBsAg. In a system of coculture of hepatic NPCs with HBV-Met, it was demonstrated that HBV (either purified virions, HBeAg, or HBsAg) suppressed the activation of NF-κB and ERK1/2,which were elicited by TLR3 and TLR4 stimulation of hepatocytes and nonparenchymal liver cells [48]. HBV blocks the MyD88 expression by an antagonistic activity of the terminal protein (TP) domain of the HBV polymerase and impairs TLR-mediated innate immune responses [49]. The HBV X (HBx) protein has also been endowed with inhibition of type I IFN induction, including interfering the virus-induced signaling adaptor (MAVS) [50] and inducing their degradation by proteasome [51].Chemically synthesized 3p-HBx-siRNA reversed HBV-induced tolerance and recovered production of type I IFNs via intrinsic activating receptor RIG-I pathway [52]. Moreover, the HBV polymerase was described to inhibit IFNβ induction via interfering the transcriptional factor DDX3 (DEAD box 3) function [53, 54].

Collectively, the persistence of HBV not only directly inhibits PRR recognition and the antiviral signaling pathways but also suppresses the frequency and function of systemic innate immune cells, resulting in systemic innate immune tolerance (Fig. 4.1).

Cell-based innate immune responses against HBV have been extensively studied in the past years. These include NK cell, NKT cells, monocyte/macrophage, as well as myeloid-derived suppressor cells (MDSCs) and mucosal-associated invariant T (MAIT) cells.

4.3 NK Cells

NK cells represent the main effector population of the innate immune system and are abundant in the human liver [55, 56]. Elegant experiments have identified NK1.1 + CD3-NK cells as the major constituent of inflammatory infiltrates in the liver, where they are enriched to approximately 30% of liver-resident lymphocytes [55]. NK cell activation is regulated by the interplay of several activating and inhibitory receptors and cytokines. The interaction between NK cells and innate immune cells such as monocyte-derived dendritic cells (DCs), plasmacytoid DCs (pDCs), and macrophages can modulate NK cell functions. NK cells depend on chemokines from KCs for recruitment and on cytokines from KCs, LSECs, and T cells for survival [57].

Whether NK cells are innocent bystanders or effective participants during HBV infection remains controversial. In acute HBV infection, the increased expression of activation markers CD69 and NKG2D and the lower levels of inhibitory markers NKG2A of NK cells indicate that NK cells were promptly activated [58]. HBV also promotes NK cell activation through TLR/IFN-α-mediated signaling pathways [59]. In the chimpanzees' liver, not only CD8 + T cells but also NK cells are the

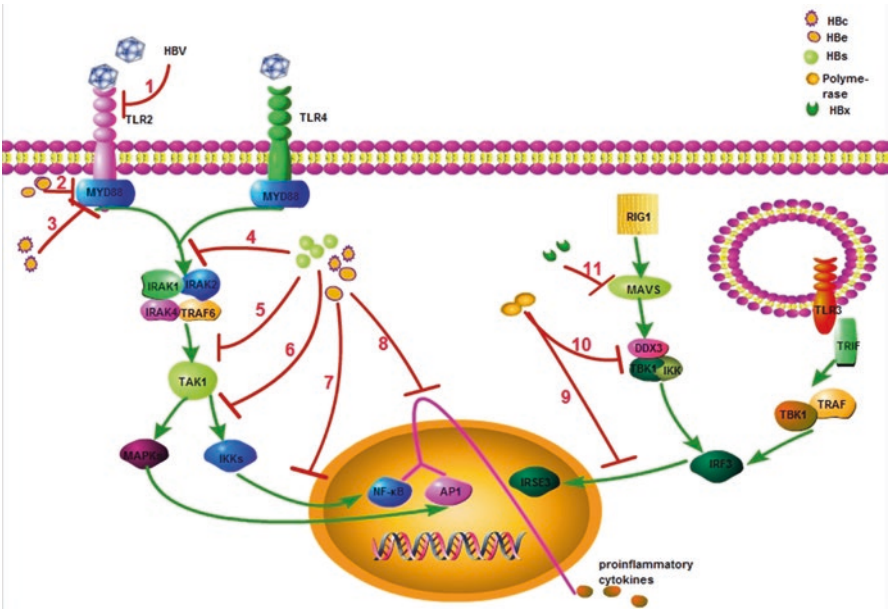

Fig. 4.1 HBV-driven inhibition of innate signaling pathways. (1)TLR2 expression is down-regulated by HBV in hepatocytes, leading to a reduced production of pro-inflammatory cytokines and chemokines; (2–3) HBeAg and HBcAg bind to MyD88 complex to inhibit TLR2 signaling; (4–6) the antiviral effect of TLR2 is dependent on the presence of molecules like TAK1, IRAK1/4, and TRAF6 and the downstream MAPK pathways which can be disturbed by HBV proteins; (7–8) HBV (either purified virions, HBeAg, or HBsAg) suppressed TLR2-mediated activation of NF-κB and MAPK signaling; (9–10) HBV polymerase inhibits the dsRNA-mediated IFN response by interfering with the IRF3 and DDX3 function; (11) HBx inhibits the dsRNA-mediated IFN response by interacting with MAVS

resource of cytokines like IFN-γ and TNF during the described non-cytopathic pre-T cell, which supports that NK cells play a non-cytopathic antiviral mechanisms to viral clearance [60]. Moreover, the activated NK cells were correlated positively with ALT levels, suggesting a close association of activated NK cells with liver necroinflammation in acute HBV infection [38]. However, some researchers show that HBV titers do not decrease at earlier time points when the frequency of NK cells increases in the blood [61]. And NK cells showed impaired function in hepatitis B infection, in which IL-10 plays an important role [38, 62]. Overall, although NK cells were activated, they might be functionally suppressed and are unable to clear the infection on their own.

Researchers have revealed the phenotype and function of NK cells during chronic HBV infection (Fig. 4.2). NK cells are defective in the production of cytokines, such as IFN-γ, impairing their noncytolyic antiviral capacity in CHB patients [63, 64]. The expression of activating receptors NKG2D and 2B4 were found to be reduced [64], meanwhile the inhibitor NKG2A [65] and T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) [66] were elevated in the NK

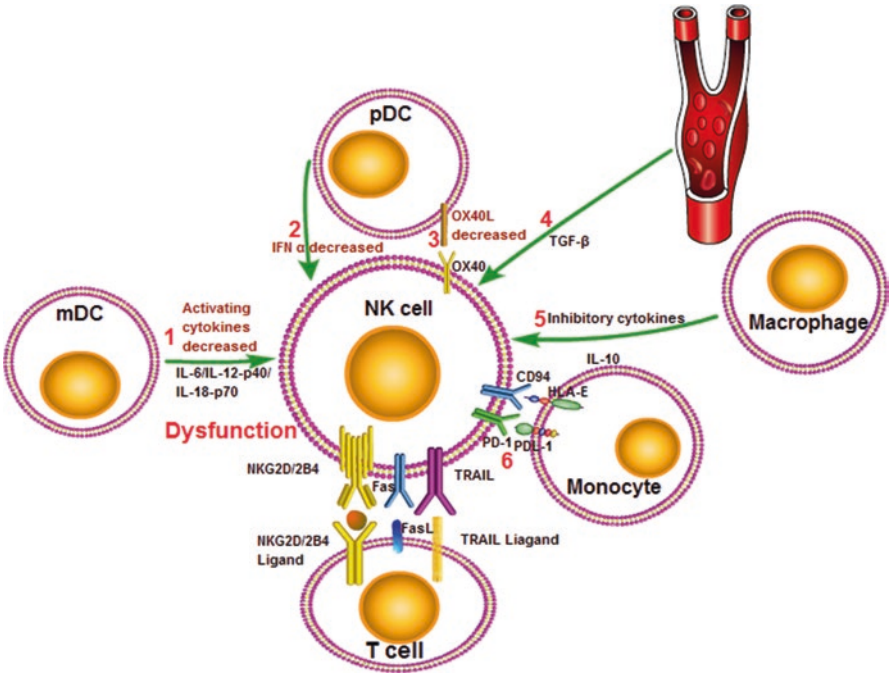

Fig. 4.2 Mechanisms of NK cells dysfunction. (1) mDCs are substantially impaired in their ability to activate NK cells via decreased activating cytokines (IL-6, IL-12, IL-18) secretion, which in turn fail to secrete adequate amounts of IFN-γ; (2) OX40L expression level and (3) the secretion of IFN-α decreased on pDCs, which make NK poorly activated through an OX40L/IFN-α-dependent pathway; (4) KCs are the main source of IL-10, which acts as an inhibitory cytokine blunting NK activation; (5) serum TGF-β level impairs NK function. (6) PD-L1 and HLA-E on suppressive monocytes educates IL-10-producing regulatory NK cells, which inhibit autologous NK-cell activation. Impairment of NK-cell-mediated cytotoxic capacity and IFN-γ production contribute to HBV persistence. The expression of activating receptors NKG2D and 2B4 was found to be reduced in the NK cells of CHB patients. Additionally, Fas and TRAIL expression on NK cells have been reported to correlate with NK-cell-mediated hepatocyte apoptosis

cells of CHB patients. And during HBV infection, both mDCs and pDCs display functional defects, which influence the cross talk with NK cells through reducing activating cytokines [67] and OX40L [68] pathways, making inefficient the NK cells activation [59]. HBsAg induces inhibitory molecules PD-L1 and HLA-E on suppressive monocytes, and the engagement of PD-L1/PD-1 and HLA-E/CD94 educates IL-10-producing regulatory NK cells, which inhibit autologous NK cell activation [62]. Impairment of NK cell-mediated cytotoxic capacity and IFN-γ production contribute to HBV persistence. Additionally, Fas [69] and TRAIL [70] receptors expression on NK cells have been reported to correlate with NK cell-mediated hepatocyte apoptosis. Intrahepatic NK cells in patients with CHB also express higher levels of PD-1, CD94, and IL-10, thereby inhibiting the activation of T cells and monocytes [62]. Schuch et al. [71] found a difference in the proportion

of memory-like NK cells, and this group of cells has special epigenetic and metabolic characteristics, in addition to the enhanced degranulation ability of CD16 stimulation, and other functions are not affected by HBV infection.

The mechanism by which NK cells control viral spread, participate in hepatic inflammatory responses, interact with intrahepatic immune cells at different stage of CHB, and associate with HBV infection outcomes remains to be further elucidated.

4.4 Liver Macrophages

KCs are tissue-resident macrophages residing in the liver. They are located in the liver sinusoids and are the largest macrophage population in the human body [72, 73]. Phenotypically, distinguishing genuine liver-resident macrophages (i.e., long-lived, self-renewing KCs originating from embryonic progenitor) from monocyte-derived macrophages can be difficult [41]. In fact, monocytes are readily recruited to the inflammatory liver, where they can differentiate into macrophages to replace apoptotic hepatocytes killed during the antiviral immune respons [74]. KCs are identified as $CD68^+$, $CD14^+$, and/or CD11b + cells (human), $ED1^+$ and/or $ED2^+$ cells (rat), and $CD68^+$, $F4/80^+$, and/or $CD11b^+$ cells (mouse) [75]. They can have opposite functions, according to physiological or pathological conditions.

There is no detailed information on the presence of HBV proteins by human KC/monocytes in vitro. However, some studies have shown that KC/monocytes may play a role in HBV elimination. Receptors like CD14 and mannose, which are shared by KC and monocytes or DCs, are known to interact with HBV proteins [76, 77]. Upon HBV exposure, release of chemokines and cytokines by KC has an indirect effect on the immune response in the liver by recruitment and activation of infiltrating leukocytes. KC cells were found to potentially attract NK and NKT cells via producing CXCL8 [78] and recruit DCs in a C-type lectins-dependent way [79] which may initiate and promote virus-specific T cell responses. In an age-related HBV mouse model, higher number of TNF-α-secreting $Ly6C^+$ monocytes and fewer IL-10-secreting KCs were thought to be associated with the elevated number of IFN-γ^+ TNF-α^+ $CD8^+$T cells and the final HBV clearance in the older cohort [80]. Additionally, KCs have been reported to express cytotoxic molecules such as TRAIL, Fas-ligand, granzyme B, perforin, and ROS, enabling them to lyse infected hepatocytes [81, 82]. However, KC may cause more damage to the organ than protective immunity provided to the host because of the antigen-nonspecific manner. Moreover, TLR3-stimulated KCs generate IFN-β suppressing HBV replication [83]. Liver KC exposed to HBV secreted IL-6 and TGF-β, leading to repression of hepatocytes nuclear factor (HNF) 1α and 4α, ending up with the interference of HBV replication partly through the activation of the mitogen-activated protein kinases (MAPK) in PHH [78, 84]. In general, KC may play a role in controlling HBV infections by inhibiting viral replication, either directly via the production of cytokines or via their interaction with other cells.

Interestingly, some other studies considered KC/monocytes play a role in systemic tolerance and liver inflammation. In contrast to other HBV particles, activated non-parenchymal cells expressed IFN, IL-6, or TNF to inhibit HBV replication [78], and KC exposed to HBV virions produced a transient increase of IL-6, IL-1β, as well as TNF-α mRNAs [78] without any induction of an IFN response. IL-10 is one of the immune-suppressive cytokines that maintains the immune tolerance during persistent HBV infection. In a mouse model, the depletion of liver KC prior to HBV infection prevented the establishment of a chronic infection [85], which correlated with IL-10 secreted by KC. In an HBV-persistent mice model, HBV could upregulate the expression of TLR2 in KCs and HBcAg-activated TLR2 producing IL-10 and suppressing CD8+ T cells for HBV persistence [86]. HBV also induced suppressive monocytes to educate NK cells produce IL-10 [62]. In an HBV replication-competent transgenic mice model, KCs contain liver immunopathology avoiding the release of HMGB1 inflammatory molecules.

So far, we are still unclear how mononuclear cells switch between phenotypes to inhibit viral replication and delay viral clearance, and further studies are required.

4.5 MDSC

To some extent, it suggested that MDSCs help protect the liver and may be beneficial for HBV replication. Although the mechanism has not been completely elucidated, HBV is likely to induce their recruitment/expansion in the liver to its own benefit. It was reported that the number of granulocytic subsets of MDSC (gMDSC) is increased in the phase of immune tolerance in the HBV natural history. Their number is inversely proportional to the degree of inflammation in the liver [87]. Some studies showed that the gMDSC expands in acute HBV in parallel with viremia, decreasing prior to the onset of liver inflammation [88]. Localized depletion of nutrients and oxygen levels in inflammatory setting can impact antiviral immunity. MDSCs were able to potently inhibit both CD4+ and CD8+ T cell responses to HBV, in a partially arginase-dependent manner [88], as well as reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS) to deplete arginine [89]. Actually, MDSCs secrete a huge amount of arginase in the liver microenvironment, which in turn lessens the amount of available arginine for lymphocyte physiology and growth. This leads to their functional inhibition [41]. Apart from starving them of arginine, another study has proposed that MDSC can regulate immunity through PD-1-induced IL-10 in CHB [90]. And MDSC-derived TGF-β and IL-10 promoted development of induced regulatory T cells (iTregs) [91]. However, RNA with 5′-triphosphate (3p-RNA) disrupted the differentiation of MDSC and Tregs, accompanied by the reduction of the immunosuppressive cytokines IL-10 and TGF-β, resulting in the improvement of immune microenvironment and inhibition of HBV replication [92]. And a latest research showed that HBeAg-induced monocytic MDSCs (mMDSCs) expansion impairs T cell function through IDO pathway [93].

4.6 NKT

NKT cells are a special subset of T lymphocytes that express surface markers of T lymphocytes (TCR) and NK cells (NK1.1) as well as CD1d-restricted molecules. NKT cells are both cytotoxic and produce a large number of cytokines such as IL-4 and IFN-γ. There are two types of NKT cells: invariant NKT cells (iNKT: expression of non-polymorphic Vα14-Jα18 TCR and activation byα-galactosylceramide (αGalCer)) and nonclassical NKT cells (expression of polymorphic TCRs and activation by non-α-GalCer molecules).

The number of NKT cells in human liver is much higher than in peripheral blood [88]. Nonclassical NKT cells can be activated by CD1d restricted recognition and then result in liver cell injury, which could thus contribute to viral pathogenesis [94]. In addition to their role in injury, activated NKT cells are known to release large antiviral cytokines such as IFNγ to inhibit HBV replication in vivo. αGalCer activates iNKT cells, which in turn activate NK cells to produce high level of IFNγ and IFNα/β, inhibiting HBV replication [55]. αGalCer-activated NKT cells can also potentiate HBV-specific CTL function, breaking the immune tolerance of CTL during HBV infection [95]. HBV-infected hepatocytes can induce activation of nonclassic NKT cells via CD1d-presented lysophosphatidylethanolamine, which activates cytokines to promote specific T-lymphocyte responses, thereby clearing the virus [96]. Some clinical studies also found the frequency decrease [97] and function decline [98] of iNKT cells in peripheral blood of patients with CHB. The functional decline of iNKT cells was closely related to the decrease in CD28 expression and the increases of Tim-3 and PD-1. In addition, clinical antiviral treatment or Tim-3 blockade [99] could partially restore the immune function of iNKT cells.

4.7 MAIT

MAIT cells are nonclassical invariant T cells that express a semi-invariant T-cell receptor specific to bacterial vitamin B2 metabolites presented by major histocompatibility complex-related protein 1 (MR1). It has been proposed that, independently of MR1 binding, MAIT cells can produce IFNɣ, TNF, and IL-17 [100] and promote antiviral immune responses. But the percentage of cytokine-producing MAIT cells were significantly reduced among HBV-infected patients relative to healthy controls [101], and their functions were seemingly impaired likely because of the increased expression of PD-1 [102] and decrease of CD69 levels [101]. Another research showed that MAIT cells isolated from HBV patients are more activated than healthy control, which can be normalized by nucleoside analog therapy [103]. Consequently, MAIT cells play an important role but their functionality is impaired in chronic HBV infection.

5 Adaptive Immune Responses

In the process of HBV infection, the cell types that can play protective or pathogenic roles form antiviral adaptive immune responses [104]. Successful control of HBV infection requires an effective expansion of the different elements of the adaptive immune system (antigen-presenting cells (APCs), $CD4^+$ T cells, $CD8^+$ T cells, Tregs, and B cells),which need to function in the liver parenchyma (Fig. 4.3) [105]. The ability to develop and maintain efficient HBV-specific adaptive immune cell networks is considered as the most important difference between HBV control and chronicity [106, 107].

5.1 Antigen-Presenting Cells

DCs are professional APCs, which express various costimulatory molecules to initiate adaptive immune responses [108]. DCs can be infected in animal models of hepadnavirus infection [109], but the large replication of HBV in DCs of patients with CHB was excluded [110, 111], and the stimulus defects are almost invisible [112–115]. Recent studies have shown that surface marker (CD80, CD83, CD86, CD40, and CCR7) expressions in pDC were related to the HBV-specific T-cell response in CHB patients. IL6ST (a subunit common to receptors in the IL-6 family cytokines) expression in pDC was identified as one cause of decreases in antigen-presenting capacity, migration capacity, and cytokine production capacity in pDC of HBV patients [116]. Adjustment of the IL6 family cytokine signaling transduction or exogenous IL-12 [114] could restore impaired function of DC.

Liver sinusoidal endothelial cells (LSECs) are organ-resident APCs capable of cross-presenting soluble exogenous antigen to $CD8^+$ T cells. Though LSECs employ similar molecular mechanisms for cross-presentation as DCs, the outcome of cross-presentation by LSEC is $CD8^+$ T-cell tolerance rather than immunity [117]. B7-1 (or B7-2) molecules can also ligate with CTLA-4 on T cells, which downregulates further T-cell activation [118]. A high and persistent load of viral antigens also have a tolerating effect [117, 119]. However, the antigen-presenting ability of LSECs was

Fig. 4.3 (continued) cytosolic mediators (TRAIL, FasL, granzyme B, perforin, ROS) promoting the lysis of HBV-infected hepatocytes. Activated DCs and B cells can initiate adaptive immune responses. (**c**). APCs can activate naive $CD4^+$ T cells to differentiate into Th1, Th2, and Thf. Th2 cells help B cells to produce anti-HBV antibodies, among which HBsAb can neutralize virus particles and also promote $CD8^+$ T-cell activation. Tfh assist B lymphocytes to produce antibodies and also promote HBV-specific $CD8^+$ T-cell response. Th1 cells can produce IFN-γ and TNFα which promote viral clearance and also help CD8+ T-cell activation. $CD8^+$ T cells promote HBV clearance through three types of pathways: direct killing of HBV-infected hepatocytes through Fas/FasL or perforin pathway, secretion of cytokines (IFN-γ and TNF) to promote HBV clearance with non-cytotoxic effects, and receptor (TRAIL)-mediated induction of apoptosis

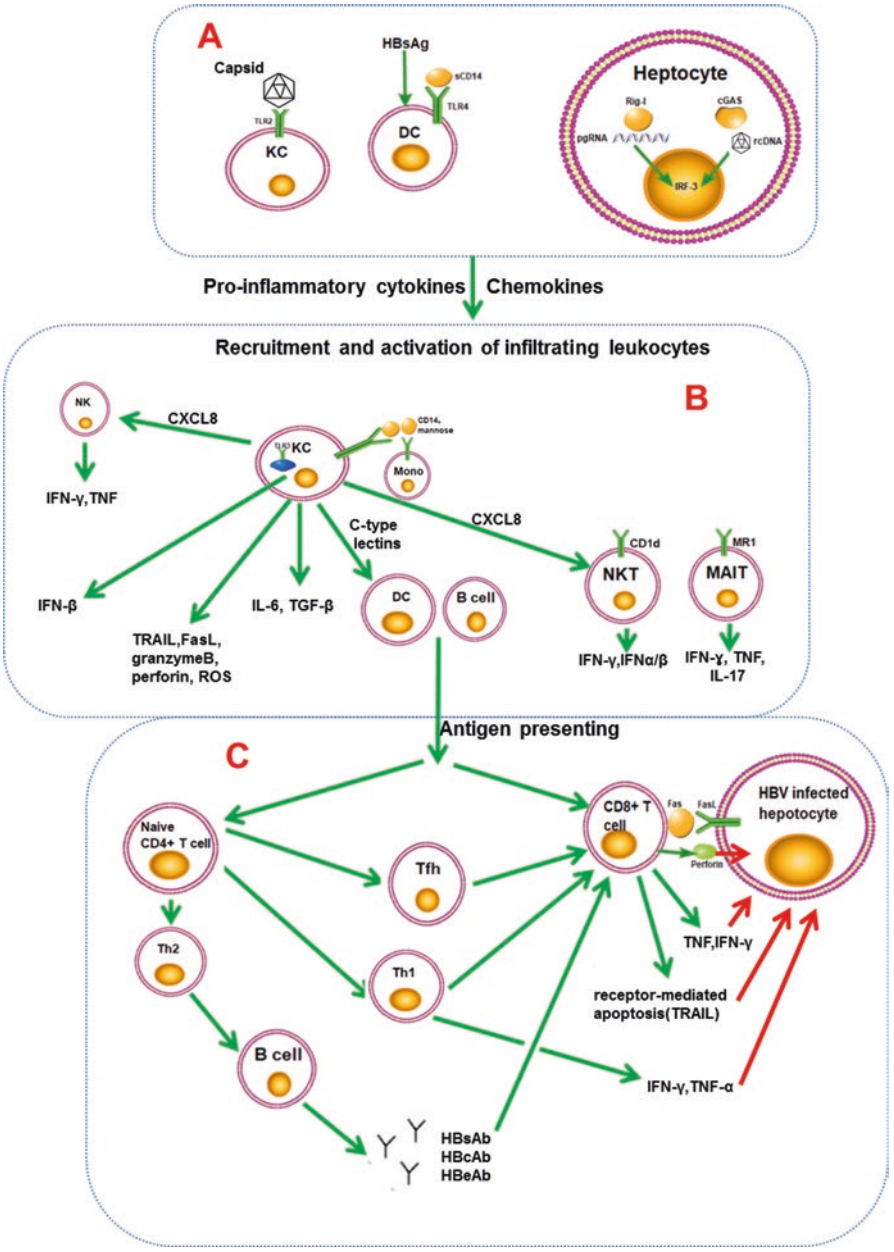

Fig. 4.3 Innate and adaptive immune response in acute HBV infection. (**a**) HBV components (HBV capsid, HBsAg, pgRNA, rcDNA) can be recognized by PRRs (e.g., TLRs, Rig-I, cGAS) in KC, DC, and hepatocytes. Activated cells secrete pro-inflammatory cytokines and chemokines (such as CXCL8, c-type lectins) to attract immune cells into the liver. (**b**) Innate immune cells can be activated either directly by HBV components or indirectly via their interaction with other cell types. Activated innate immune cells secrete pro-inflammatory factors (IFN-α/β, IFN-γ, TNF, IL-6, IL-17, etc.) to promote HBV clearance through non-cytolytic pathways and also secrete

enhanced by NOD1 agonists (DAP) application, which resulted in enhanced T-cell responses and inhibited HBV replication in a mouse model [120].

5.2 *CD4⁺ T Cells*

More and more studies have shown that the immune response of CD4⁺T lymphocyte plays an important role in the outcome of HBV infection. The early CD4 ⁺ T-lymphocyte response in HBV infection is accompanied by a virus-specific CD8 ⁺ T-lymphocyte response and is an important factor in the eventual elimination of the virus. HBV-specific CD4⁺ T lymphocytes can be detected in peripheral blood of patients with acute HBV infection; while in patients with chronic HBV infection, CD4 ⁺ T-lymphocyte response is significantly absent in the acute phase of infection. The CD4⁺T cells specific to HBV nucleocapsid [118, 121, 122, 123], envelope [124, 125], polymerase [126, 127], and x [128] proteins were induced after HBV infection.

Studies have shown that in chimpanzees infected with HBV, CD4⁺ T lymphocytes are knocked out by antibodies when the viremia reached its peak, and virus clearance and liver inflammation are not affected [129]. However, CD4 ⁺ T-lymphocyte knockout before HBV infection resulted in persistent HBV infection and a significant decrease in the number and function of HBV-specific CD8 ⁺ T lymphocytes [17, 130]. CD4+ T cells produced a large number of cytokines, which were necessary for the effective development of effector cytotoxic CD8 T cells and B-cell antibody production [131]. Therefore, although CD4⁺ T lymphocytes are not directly involved in virus clearance and tissue injury, they assist in the activation of HBV-specific CD8 ⁺ T lymphocytes, which may also be the reason why chronic infection with HBV is more likely to occur after HIV and HBV coinfection.

In addition, studies have reported that CD4 ⁺ T lymphocytes have a direct inhibitory effect on the virus. For example, CD4 ⁺ T lymphocytes with a helper T-lymphocyte (Th) 1 functional phenotype have a direct antiviral effect and immunopathogenic effects in the HBV transgenic mouse model [132]; application of HBV antigen-specific T lymphocytes in vitro experiments with cell clones revealed that HLA class II-restricted CD4 ⁺ CTLs are directly involved in the immune clearance of HBV-infected cells and immune damage in hepatocytes [133]. In HBV-carrier mice, the therapeutic vaccine immunization could induce a very effective CD4 ⁺ T-cell immune response, and large numbers of HBs-specific CD4⁺T-cell-secreting IFN-γ, interleukin (IL)-2 , and TNF-α were found in the liver [134]. Moreover, CD4 T cells can regulate the immune inflammatory response by secreting pro- (CXCL-8, IL-17) or anti-inflammatory (IL-10) cytokines [135].

With the deepening of research, the division of CD4⁺ T-lymphocyte subsets has become more and more detailed according to the differences in cytokine secretion, transcription factors, and homing receptor expression. HBV-specific CD4⁺

T lymphocytes are mostly Th1 subpopulations and express the chemokine receptor (CXCR-3). Correspondingly, hepatocytes, hepatic stellate cells, hepatic sinusoidal cells, and KCs produce CXCR3 ligands, which play a role in recruiting effector T lymphocytes to the liver. Recruited T-lymphocyte subsets and their activation status in the liver affected the outcome of viral infection. Studies have shown that IFN-γ secreted by lymphocytes in HBV infection induces KCs to produce CXCL9 and recruits HBV-specific CD4 + T lymphocytes to enter the liver for apoptosis, leading to chronic HBV [136].

In addition to the Th1 subsets, Treg, Th17, and follicular helper cells (Tfh) were also involved in the regulation of HBV clearance and liver inflammation.

Tregs are a special subset of $CD4^+$ T cells that expressed various markers such as CD25 (IL-2 receptor α chain), CTL antigen 4 (CTLA-4), and the forkhead family transcription factor (FoxP3) [137] and played a negative regulatory role in HBV infection. Early studies have shown that Treg inhibited anti-HBV immunomodulation through intercellular contact or production of regulatory cytokines like IL-10 [138]. Clinical studies have shown that circulating Tregs modulate the function and expansion of HBV-specific CD8+ cells ex vivo in all patients, regardless of whether they have chronic or resolved HBV infection [139]. Depletion of CD4 + CD25+ cells increased the function of HBV-specific T cells in vitro [140, 141]. Recent studies have found a subset natural Treg that infiltrates the liver in HBV infection without further activation or expansion but still interferes with T-cell-mediated virus clearance [142]. In a mouse model of acute HBV infection, researchers found that Tregs mitigated mediated liver damage by downregulating the antiviral activity of effector T cells by limiting cytokine production and cytotoxicity. Furthermore, Tregs controlled the recruitment of innate immune cells such as macrophages and DCs to the infected liver [143].

Th17 lymphocyte is a pro-inflammatory cell subset. The number and frequency of Th17 lymphocytes in patients with chronic HBV infection or HBV-related acute-on-chronic liver failure were significantly higher than those in control group; the levels of Th17 in peripheral blood and liver were positively correlated with serum viral load, transaminase level, and histological activity index [144]. Studies indicated that cytokines secreted by Th17, including IL-17, IL-21, and IL-22, mediated inflammation and fibrosis after liver injury [135].

Tfh lymphocyte subset specifically assists B lymphocytes to produce antibodies. Studies have also shown that Tfh promoted the HBV-specific CD8 + T-cell response by producing IL-21, which was contributed to virus clearance [145]. In CHB patients with HBeAg serological conversion, the frequency of Tfh in peripheral blood was significantly higher than that in HBeAg-positive patients; and for CHB patients receiving antiviral therapy, the frequency change of Tfh has a predictive value for antiviral therapy [146]. In studies of HBV-infected mice and blood from patients with chronic HBV infection, a Tfh-cell response to HBsAg was required for HBV clearance and that this response was blocked by Treg cells [147].

5.3 *CD8⁺ T Cells*

CD8 + T lymphocytes played a very important role in the control of HBV replication and immune-mediated liver injury. The effector functions of CD8⁺ T lymphocytes include direct killing through Fas/FasL or perforin pathway, secretion of cytokines (IFN-γ and TNF) to promote HBV clearance with non-cytotoxic effects, and receptor-mediated induction of apoptosis (e.g., through TRAIL) (Fig. 4.4).

HBV-specific CD8 ⁺ T lymphocytes appear in peripheral blood of acute HBV infection, showing an activated phenotype, but their proliferative capacity is decreased; as the infection is restored, the proliferation of CD8 ⁺ T lymphocytes is also restored [38, 129, 148, 149]. After the recovery of acute HBV infection, the number and frequency of HBV-specific CD8⁺T lymphocytes decreased accordingly. However, memory T-lymphocyte responses can still be detected 10–30 years; virus titer remained at high levels [150].

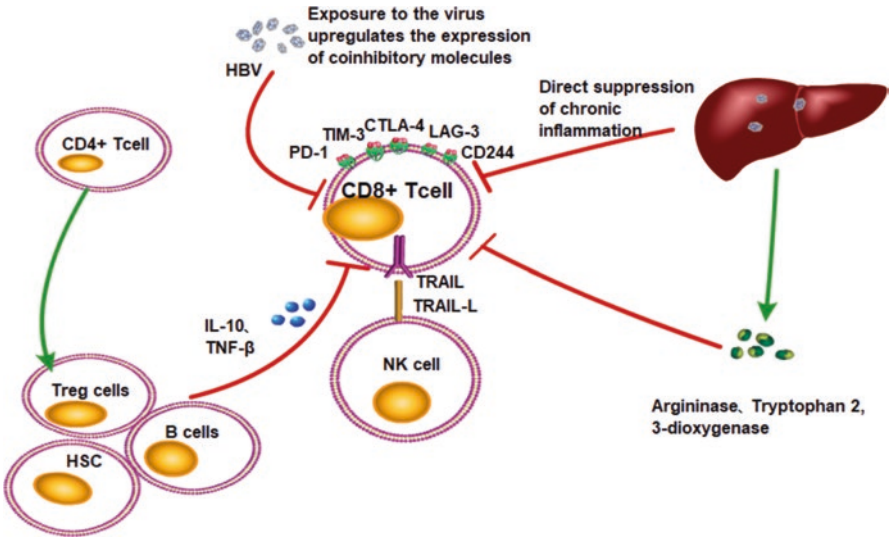

Fig. 4.4 Mechanisms of CD8⁺ T-lymphocyte dysfunction in chronic HBV infection. (1) Chronic hepatic inflammation inhibited the entry of HBV-specific T cells into liver tissue and also inhibited the function of intrahepatic T lymphocytes and the ability of hepatocytes to respond to viral cytokines. (2) Long-term exposure of T lymphocytes to HBV antigens leads to T-lymphocyte depletion and high expression of co-inhibitory molecules PD -1, TIM-3, CTLA −4, and CD244174, (3) The upregulated expression of TRAIL receptor on the surface of depleted T lymphocytes interacted with the TRAIL molecule expressed on the NK cell and promoted the apoptosis of HBV-specific CD8 + T lymphocytes. (4) The liver environment of chronic HBV infection also plays an important role in the function of CD8 + T lymphocytes. Damaged liver cells release various enzymes, such as arginase and tryptophan 2, 3-dioxygenase. (5) Treg, B lymphocytes, and hepatic stellate cells can also secrete the inhibitory cytokine IL-10, negatively regulating the function of HBV-specific T lymphocytes

The frequency and response level of virus-specific CD8 + T lymphocytes in patients with chronic HBV infection were significantly lower than those in patients with acute HBV infection, which was manifested as the absence or attenuation of virus-specific $CD8^{+}$T-lymphocyte response, leading to persistent HBV infection [151–155]. Meanwhile, persistent infection can also cause T lymphocytes to gradually lose their ability to proliferate and lose the ability to produce antiviral effector molecules. Studies showed that circulating multi-specific HBV-specific CD8 + T-cell responses were primarily detectable in vitro in patients with low viral load. In individuals with high levels of HBV replication, it was difficult to detect virus-specific CD8 + T cells even after in vitro amplification [154]. HBV-specific CD8 + T cells could not or were not sufficiently activated by APC and therefore lost most of their ability to proliferate and produce cytokines when exposed to antigens, like IFN-γ [155].

The mechanisms involved in CD8+ T-lymphocyte failure include: (1) Chronic hepatic inflammation can inhibit the entry of HBV-specific T cells into liver tissue [156] and also inhibit the function of intrahepatic T lymphocytes and the ability of hepatocytes to respond to viral cytokines [157]. (2) Long-term exposure of T lymphocytes to HBV antigens lead to T-lymphocyte depletion [158, 159]and high expression of coinhibitory molecules PD -1 [155, 160], TIM – 3 [161, 162], CTLA −4 [163], and CD244 [164]. More than 90% of infiltrating HBV-specific CD8 + T lymphocytes express PD-1 and CD244; blocking the function of co-suppressive molecules such as PD-1 can significantly enhance the virus-specific CD8 + T lymphocyte response and achieve continuous inhibition of viral replication [165]. (3) The upregulated expression of TRAIL receptor on the surface of depleted T lymphocytes interacted with the TRAIL molecule expressed on the NK cell and promoted the apoptosis of HBV-specific CD8 + T lymphocytes, which further led to the loss of HBV-specific CD8 + T lymphocytes [70]. (4) The liver environment of chronic HBV infection also plays an important role in the function of $CD8^{+}$ T lymphocytes [87]. Damaged liver cells release various enzymes, such as arginase and tryptophan 2, 3-dioxygenase; infiltrating cells in the liver, such as myeloid inhibitory cells, can also produce arginase, which consumes a large amount of amino acids, including arginine and tryptophan, resulting in the dysfunction of T-lymphocyte maintenance. (5) Treg, B lymphocytes, and hepatic stellate cells can also secrete the inhibitory cytokine IL-10, which also negatively regulates the function of HBV-specific T lymphocytes. In summary, many factors work together to cause defects in CD8 + T-lymphocyte function, and different patients may behave differently due to differences in viral load and liver inflammation.

5.4 B Cells

B-lymphocyte response is a neglected part in hepatitis B research. The knowledge about B-lymphocyte response in HBV infection mainly comes from the detection of HBV serological markers, which mainly reflects the humoral immunity against

different protein components of the virus, including nucleocapsid, envelope, polymerase, and HBx protein. Different antibodies against HBV, particularly antibodies specific for envelope antigens and nucleocapsid antigen, can be used to distinguish different clinical stages of HBV infection [166]. Anti-HBc appeared during the acute phase of infection, could coexist with hyperviremia, and may persist for life after clinical cure; therefore, it is a hallmark of HBV infection or infection [167]. Anti-HBc levels in serum can be used as a predictor of treatment response in CHB patients. The emergence of anti-HBe is much later than anti-HBc, and the emergence of anti-HBe often indicated a better prognosis. HBV clearance was associated with the production of anti-HBs [168], and high levels of anti-HBe antibodies could control HBV infection [169].

Some studies suggested that the production of antibodies in chronic HBV infection was T-lymphocyte dependent [170]. Anti-HBs generation disorder in chronic HBV infection was mainly caused by CD4 + Foxp3-type I Treg from the liver, which could hinder the maturation of HBV-specific B lymphocytes and Tfh in germinal center.

Anti-HBs are neutralizing antibodies to HBV and are considered to be protective antibodies. Anti-HBs could form complexes with virus particles that prevented the virus from entering uninfected liver cells. Antibodies against preS1 neutralized the virus and prevented it from infecting liver cells [171]. The presence of anti-HBs antibodies was a sign of virus control and disease recovery. After the recovery of acute HBV infection, HBV-neutralizing antibodies also played an important role in long-term virus control [168, 169]. Studies have shown that administration of HBV-neutralizing antibody in HBV mouse model not only decreased HBsAg level but also promoted the response of HBV-specific T lymphocytes [172]. This suggested that HBV-neutralizing antibodies could not only prevent HBV from entering uninfected hepatocytes but also recognize HBV-infected hepatocytes and induce antibody-dependent cytotoxicity.

In the process of HBV infection, B lymphocytes not only play a role of producing antibodies but also play a role of antigen presentation and immune regulation that assist T-lymphocyte activation [173]. HBc-specific B lymphocytes can effectively present antigen to T-helper cells. In patients with chronic HBV and HCV coinfection, the proportion of B lymphocytes with an activated phenotype increased, but their proliferative capacity decreased significantly [174]. The elevated proportion of regulatory B lymphocytes producing IL-10 in CHB patients was associated with liver inflammation, and this group of B lymphocytes can inhibit the response of virus-specific CD8 + T lymphocytes by secreting IL-10 [175]. But anti-HBV-specific B cells localized to the liver are involved in the increase of HBs/anti-HBs immune complex formation during CHB exacerbations [176, 177]. In recent years, due to the optimization of detection methods, it is possible to detect HBV antigen-specific B cells in the peripheral blood and liver. HBsAg-specific B cells from patients with CHB, which had an accumulation of CD21–CD27-atypical memory B cells (atMBC) with high expression of PD-1, had defective antibody production, consistent with undetectable anti-HBs antibodies in vivo [178]. Blocking PD-1 also partially restores B-cell function [179]. In general, studies on the immune response

and function of B lymphocytes infected with HBV are relatively inadequate. It is necessary to further explore how B-lymphocyte mediated interaction between humoral immunity and cellular immunity after HBV infection.

6 Liver Injury in HBV Infection

In addition to immune-mediated liver damage, various proteins and nucleic acid components of HBV directly cause damage to liver cells and participate in the pathogenesis from chronic hepatitis and liver fibrosis to hepatocellular carcinoma. This suggests that the development of direct antiviral drugs for the life cycle of HBV can slow the progression of end-stage liver disease caused by chronic HBV infection (Fig. 4.5).

6.1 HBV-Induced ER and Mitochondrial Dysfunctions

Under normal conditions, the endoplasmic reticulum (ER) and mitochondria have protective mechanisms to restore their damaged function by intrinsic or extrinsic stresses. However, HBV can cause ER and mitochondrial dysfunction, which interferes with cell homeostasis and causes liver damage [180].

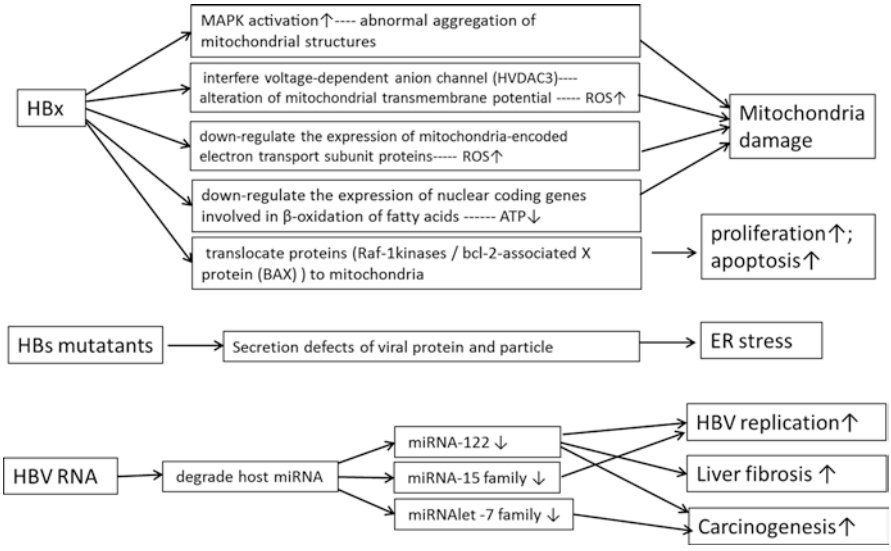

Fig. 4.5 HBV components directly cause damage to liver cells. *MAPK* p38 MAPK, *HVDAC3*: voltage-dependent anion channel, *ROS* reactive oxygen species, *ATP* adenosine triphosphate, *ER* endoplasmic reticulum, *BAX* bcl-2-associated X protein

Mitochondria play a key role in cellular energy, calcium and REDOX balance, cell signaling, regulation of cell metabolism and cell death, and thermogenesis [181]. The most prominent function of mitochondria is the synthesis of cellular energy, adenosine triphosphate (ATP), which provides the chemicals for metabolism and the reactants for signaling pathways [182]. In HBV infection, HBx protein is associated with mitochondrial aggregation or damage. By inducing p38 MAPK activation, HBx proteins cause increasing microtubule-dependent dynein activity, leading to abnormal aggregation of mitochondrial structures around the nucleus, ultimately leading to cell death [183, 184]. Another possible mechanism is to interact with the voltage-dependent anion channel (HVDAC3), which is known as mitochondrial porins [185], to cause the alteration of mitochondrial transmembrane potential leading to generation of ROS [186].

HBx protein can also affect mitochondrial function by regulating gene expression. HBx downregulates the expression of mitochondria-encoded electron transport subunit proteins in oxidative phosphorylation, and the damage of electron transport leads to high levels of cell ROS [187]. In addition, HBx downregulates the expression of nuclear coding genes involved in β-oxidation of fatty acids, causing deficiency of energy source and leading to low level of ATP [188]. HBx protein can also affect cell fate by translocating a series of proteins to mitochondria. Raf-1 kinases involved in the Ras-induced MAPK pathway can be translocated to mitochondria by HBx, leading to hepatic cell proliferation [189], whereas apoptosis regulator bcl-2-associated X protein (BAX) being translocated to mitochondria leading to cell apoptosis [190]. These evidences suggest that HBx-induced mitochondria dysfunction contributes to the HBV-induced liver injury.

ER, as a sensor of intracellular or extracellular stimulation, is crucial to cell homeostasis. ER can be divided into rough ER and fine ER, which have different functions. The rough ER is involved in synthesis, folding, and glycosylation of secretory or integral membrane proteins [191] and is well developed in specialized secretory cells such as hepatocytes. The smooth ER is involved in several metabolic processes including the synthesis of lipids (phospholipids and steroids), metabolism of carbohydrates, regulation of calcium concentration, and detoxification of drugs [192]. Under strict quality-control process (QCR), appropriate protein folding and modification are the most important and characteristic functions of ER [193]. QCR means that only the right mature protein will be exported to the golgi complex, and the misfolded protein will remain in ER to complete this process or degrade the protein [194]. Viral infection induces the synthesis of a large number of viral proteins, resulting in protein overload in ER. Unfolded or misfolded proteins induced by stimulation accumulate and aggregate in ER, leading to ER stress.

In HBV-infected cells, a large number of HBV surface proteins are synthesized and folded in ER during their productive life cycle, often leading to disturbance of ER homeostasis and ER stress. This became known by identifying cell-accumulating ER mutant surface proteins (pre-s1 and pre-s2 mutants), known as ground-glass hepatocytes (GGH) representing ER hypertrophy [195, 196]. HBV-activated ER stress can lead to the expression of ER degradation enhancers. Mannosidase 1 (EDEM1) participates in the ERAD pathway by activating the IRE1/XBP1 pathway,

limiting the amount of surface proteins to alleviate ER stress and protect cells [197]. In fact, rhythmic or transient ER stress is the protective mechanism of cell survival, but chronic ER stress in chronic HBV infection and other pathological conditions can cause various liver diseases. Therefore, ER stress is crucial for correct viral protein folding and HBV replication.

6.2 *HBV Surface Antigen Mutations in Fulminant Hepatitis B and Occult HBV Infection*

A single open reading frame (S-ORF) expresses three surface proteins: large surface antigens (LHBs), middle surface antigens (MHBs), and small surface antigens (SHBs). LHBs are encoded by the 2.4 kb subgenomic RNA, and MHBs and SHBs are encoded by the 2.1 kb subgenomic RNA. Subgenomic RNAs of 2.4 kb and 2.1 kb are driven by preS1 and preS2/S promoters, respectively.

Defective surface antigen mutations are frequently detected in patients with chronic HBV infection [198–203], which often lead to HBV infection despite the presence of protective anti-HBs antibodies [204]. Defective surface antigen expression, such as specific mutations of preS/S gene, may lead to secretion defects of viral proteins and particles, the accumulation of virus products in liver cells, ER stress, and finally liver cell injury [198].

HBV surface antigen variants isolated from patients with HBV-associated FH suggest that defective surface antigen expression may play a role in the pathogenesis of FH [198, 199, 205–209]. Defective surface antigen expression has been shown to increase HBV replication [199, 210]. The deficiency of hepatotropic DNA virus envelope protein can lead to the accumulation of cccDNA or the ultimate death of infected hepatocytes through direct cytopathic effect [211–213]. The increase of cccDNA level can also promote HBV replication. Defective secretion and increased replication of virus particles may aggravate FH [214]. Intracellular accumulation of viral products may also result in liver injury through abnormal activation of CTL responses [210]. In addition, mutations associated with defective surface antigen expression can result in the loss or alteration of B- and T-cell epitopes in preS1 and preS2 proteins [106].

Occult HBV infection (OBI) is characterized by very low HBV DNA levels in plasma and/or liver of individuals with HBV surface antigen (HBsAg) negative and hepatitis core antigen antibody (anti-HBc) positive/negative [215]. Defective surface antigen expression mutations may be involved in OBI. Point mutations and deletions as well as insertion mutations occur in OBI, and mutations in preS/S genes are the most widely studied [216]. MHR mutations observed in individual OBI strains [217–220] can significantly reduce the sensitivity of commercial HBsAg immunoassays and impair the secretion of virions and/or S proteins [219]. PreS/S mutation in OBI changed the transcription of 2.4 kb and 2.1 kb HBV RNA and then changed the ratio of LHBs/MHBs/SHBs proteins [216]. These mutations can directly reduce the level of surface antigens, leading to the retention and accumulation

of HBsAg in cells, and weaken the secretion of HBsAg by changing the LHBs/MHBs/SHBs protein ratio [200, 201, 221]. Because of these reasons, the level of HBsAg in peripheral blood is very low and even undetectable. It was speculated that defective surface-antigen-associated mutations might represent novel biomarkers for OBI.

6.3 HBV RNAs Deregulate miRNA Functions Leading to Pathological Consequences

RNAs, particularly noncoding RNAs, have diverse functions [222]. Viral RNA not only serves as a template for protein synthesis and viral DNA replication, but also shows biological functions involved in its pathogenesis [223, 224]. Even if HBV DNA is maintained at relatively low level by nucleoside analogues, free viral RNA may damage the host, leading to cirrhosis or HCC. MicroRNAs (miRNAs) are short, single-stranded, noncoding RNAs. Mature miRNAs are recruited into the Ago2-related RNA-induced silencing complex (RISC) and act to suppress the gene expression of target mRNAs. HBV RNA can be pathogenic by binding to and degrading miRNAs.

MiR-122 negatively regulates HBV replication depending on the expression level of cyclin G1 [225]. In HBV-producing cells and liver tissues from CHB patients, the expression of miR-122 is decreased [225–227]. Since the expression levels of pri-miR-122 and pre-miR122, the precursors of miR-122, were not decreased in HBV-positive HCC tissues and cells [226, 228, 229], it is thought that the mechanism by which miR-122 is downregulated may be that HBV RNA sequesters to it and causes its degradation. The upregulation of cyclin D1, a member of miR-15 family, was demonstrated to be required for HBV replication [230]. And HBV RNA can also sequester miR-15a and miR-16-1 to downregulate their expression. miR-122 inhibits fibrosis by blocking collagen synthesis via the TGF-β pathway. The decrease of miR-122 expression caused by HBV RNA leads to the development of liver fibrosis through the activation of collagen synthesis [231]. miR-122 also has a role in inhibit carcinogenesis. Decreased miR-122 levels resulted in enhanced proliferation and invasiveness of HCC in vitro and tumorigenicity in vivo [226]. HBV may also bind other miRNAs to promote viral replication, such as miR-15 family [230], or promote tumorigenesis such as miR-let-7 family [223, 232].

6.4 HBV X Protein Interferes with the Apoptosis Signaling to Promote Viral Proliferation and HCC Progression

At present, the relationship between HBV infection and apoptosis is still uncertain. Among all HBV proteins, HBx plays a major role in the interference of apoptosis. The mainstream view is that HBx can inhibit cell apoptosis, thereby promoting the

proliferation of viruses and promoting the progression of HCC [233–235]. In order to persist in host cells for a long term, HBV can inhibit cell death by activating oncogenes or disrupting signaling pathways, thereby promoting HCC progression. HBV can also inhibit apoptosis and promote the development of HCC by upregulating some growth-promoting proteins, such as cationic amino acid transporter 1 (cat-1) [236]. In patients with chronic HBV infection, a decrease in infected hepatocyte apoptosis may also be due to attenuated CTL responses [237, 238]. In chronic HBV infection mice model, cIAPs attenuated HBV elimination and HBV-infected hepatocyte death relied on TNF [239], while inhibition of cIAPs or cIAPs silencing reversed the phenomenon [240, 241]. On the other hand, promotion of apoptosis by HBV has also been reported [190, 242–245]. The reason for the inconsistent effect of HBV on apoptosis is not clear, but it may be due to different experimental conditions or different HBV genotypes used in different laboratories. Although induction of apoptosis is thought to be antitumor, apoptotic cells can promote the proliferation of surrounding tumor cells by altering the microenvironment [246, 247].

7 Immune-Tolerant Phase of HBV Infection

The immune-tolerant (IT) phase occurs in the early stage of hepatitis B infection which is characterized by high level of viral replication but no liver inflammation [248]. It is believed for a long time that no change in liver transaminase means no HBV specific T-cell responses [129]. In fact, in CHB patients, serum transaminase levels are not directly proportional to the quantity of circulating and intrahepatic HBV-specific T cells [151, 154]. Studies in adolescents in the IT phase have found that HBV-specific T cells are present in these adolescents, and these HBV-specific T cells are superior in quantity and function to CHB patients in the “immune clearance” phase. Moreover, normal ALT levels during the IT phase do not mean that the liver has no inflammation or fibrosis. Liver biopsy results in patients in IT phase showed mild liver inflammation and fibrosis [249, 250], indicative of immune-mediated liver damage and the destruction of hepatocytes. The limited killing of HBV-infected hepatocytes [249, 251] and the immunological escape of HBV [252] exist during this period. However, strong inflammatory response in the liver related to elevated ALT is associated with intrahepatic granulocytes, monocytes, and non-specific T-cell aggregation [151, 253–255].Therefore, “immune tolerant” phase is different from “tolerance” in immunology, during which the immune system still recognizes antigens and responds.

8 Age-Dependent Immune Response to HBV Infection

Ninety percent of neonates with vertical transmission or perinatal infection with HBV can cause chronicity [256]. This is thought to be related to the defects of the neonatal immune system to fail to prime effective HBV-specific T- and B-cell

responses [145]. Neonatal T-cell response is biased toward Th2/Treg-type response, gradually shifting toward Th1-type response as age increases [257].The effector memory T-cell pool is gradually increased, which is more effective in responding to cognate infection and more sensitive to cytokine-mediated activation [258]. Splenocytes of adult C57BL/6 mice were adoptively transferred to young or adult $Rag1^{-/-}$ mice carrying HBV, and adult mice could produce higher levels of IL-21, which is critical for T- and B-cell responses [145]. And the age-dependent expression of another chemokine, CXCL13, which is important for B-cell trafficking and lymphoid architecture and development, was proven to be important in directing an effective immune response against HBV [259].

This age-related HBV persistence may also be due to differences in gut microbiota. Gut microbiota can affect local and systemic immunity [260, 261]. C3H/HeN mice gut microbiota was found to be stabilized at 9 weeks of age [262]. Introducing AAV-HBV DNA into 12-week-old mice led to clearance of HBV DNA within 6 weeks. But when the gut microbiota of mice was removed with antibiotics, the humoral immunity, cellular immunity, and anti-HBV effects of the mice were impaired [262]. However, introducing AAV-HBV DNA into 6-week-old mice caused persistence of HBV up to 26 weeks. This phenomenon was thought to be caused by activation of TLR4 on the KCs after binding to the LPS, which is produced primarily by Gram-negative bacteria [262].

9 Maternal Effect on HBV Persistence: Induction of Trained Immunity in Human Neonates of HBV^+ Mothers

HBV carrier mothers can influence the immune system of their offspring, thus affecting the outcome of HBV infection in the child [263]. HBV heterozygous female mice were crossed with wild-type male mice to produce HBV-negative mice. Introducing 1.3mer HBV genomic DNA into livers of these mice at 9 weeks old (adult mice) causes HBV DNA replication for up to 28 weeks. While introducing 1.3mer HBV genomic DNA into age-matched mice with HBV-negative mothers, HBV DNA was cleared 4 weeks later. Mechanistic studies indicated that the expression of programmed death 1 (PD-L1) in HBV-specific CD8+ T cells and KCs in offspring of HBV-positive mother mice was upregulated, resulting in defective CD8+ T-cell response. Blocking PD-L1 binding to its ligand (PD-1) or depletion of KCs could restore CTL function and promote HBV clearance [263].

From a viral perspective, the presence of HBeAg in HBV-positive mothers and offspring was critical for chronic HBV progeny [263]. In 1973, Okada et al. already found that maternal HBeAg positivity was associated with HBV carrying of offspring [264]. Further studies have shown that offspring macrophages were regulated by maternal HBeAg. The offspring macrophages underwent M2 polarization to anti-inflammatory when encountered with HBeAg again after birth [263]. However, in the offspring of HBV-negative mother mice, macrophages underwent M1 pro-inflammatory polarization when stimulated by HBeAg or other HBV antigens [263].

HBeAg can cross the placenta [265]. Therefore, the regulation of macrophages by maternal HBeAg may occur in utero [266]. KCs mature in the fetal liver and can maintain themselves until adulthood without the need for circulating monocyte supplementation [267]. In addition, HBV-positive mothers may also achieve the regulation of their HBeAg on progeny KCs by breastfeeding [268]. Hong et al. also found that HBV can regulate the immune system of the offspring in utero through studies of the umbilical cord blood in offspring of HBV-positive and healthy mothers. HBV exposure in utero led to lower IL-10 and higher IL-12p40 and IFN-α2, maturation and activation of CD14+ monocytes enhancement, stronger Th1 polarization response, and better immune responses to unrelated pathogens [269]. These results indicate that the maternal HBV can regulate the neonatal immune system and lead to HBV persistence.

10 Conclusions

CHB is an immune-mediated disease in which the interaction between HBV and innate immune responses and adaptive immune responses determines the outcome of the infection. Due to the complexity of HBV and immune regulatory networks, more detailed and in-depth research is needed to fully clarify the mechanisms of chronic HBV infection in order to obtain more precise and specific treatment strategies. At present, the understanding of the relationship between the chronicity of HBV infection and the dysfunction of innate immunity and adaptive immunity is becoming more and more profound. The treatment strategies and drugs designed for the corresponding mechanisms bring hope to the clinical cure of CHB infection, such as TLR7 or RIG-I agonists for enhancing innate immune responses, as well as new immunotherapeutic strategies such as PD-1 blockade, therapeutic vaccines, and chimeric antigen receptor T lymphocytes that enhance adaptive immune responses. This will further deepen the understanding of immune pathogenesis of HBV infection and is expected to eventually achieve the cure of the disease.

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Chapter 5
Cell Culture Models and Animal Models for HBV Study

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Abstract Highly representative and relevant cell and mouse models are required for HBV study, including uncovering its lifecycle, investigation of the viral-host interaction, and development and evaluation of the novel antiviral therapy. During the past 40 years, both HBV cell culture models and animal models have evolved over several generations, each with significant improvement for specific purposes. In one aspect, HBV cell culture models experienced the original noninfection model including HBV plasmid DNA transfection and HBV genome integrated stable cells such as HepG2.2.15 which constitutively produces HBV virus and HepAD38 cells and its derivatives which drug-regulated HBV production. As for HBV infection models, HepaRG cells once dominated the HBV infection field for over a decade, but its complicated and labor-extensive cell differentiation procedures discouraged primary researchers from stepping in the field. The identification of human NTCP as HBV receptor evoked great enthusiasm of the whole HBV field, and its readily adaptive characteristic makes it popular in many HBV laboratories. Recombinant cccDNA (rc-cccDNA) emerged recently aiming to tackle the very basic question of how to eventually eradicate cccDNA without HBV real virus infection. In the other aspect, HBV transgenic mouse was firstly generated in the 1990s, which was helpful to decipher HBV production in vivo. However, the HBV transgenic mice were naturally immune tolerant to HBV viral products. Subsequently, a series of noninte-

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grated HBV mouse models were generated through plasmid hydrodynamic tail vein injection and viral vector-mediated delivery approaches, and HBV full life cycle was incomplete as cccDNA was not formed from HBV relaxed circular DNA (rcDNA). Human NTCP transgenic mouse still could not support productive HBV infection, and humanized mouse liver with human hepatocytes which supported whole HBV life cycle still dominates HBV infection in vivo, a value but expensive model until now. Other methods to empower mouse to carry HBV cccDNA were also exploited. In this chapter, we summarized the advantages and disadvantages of each model historically and provided protocols for HBV infection in HepG2-NTCP cells, HBV rc-cccDNA transfection in HepG2 cells, and HBV infection in NRG-Fah−/− liver humanized mouse.

1 Introduction

Human hepatitis B virus (HBV) is one member of the *Hepadnaviridae* family, which contains a small partial complementary double-strand relaxed circular DNA (rcDNA) genome of approximate 3200 base pair (bp) in length. One characteristic of HBV is its species-specific and tissue-specific infection of the human hepatocyte. HBV infection often causes acute and chronic hepatitis and later, with a large portion, will gradually progress from no symptomatic liver disease to fibrosis and finally, after decades, to human hepatocellular carcinoma (HCC) [1]. The worldwide prevalence of chronic HBV infection was estimated to be 3.5% of the whole population with 257 million people living with chronic infection in 2016, who are at an increased risk to advance to cirrhosis and HCC if were not properly managed and medically cared [2, 3]. Clinically, HBV treatments are classified into two categories based on different mechanisms. The major category is nucleos(t)ide analogs (NAs) which directly work on the virus itself by inhibiting reverse transcription from pgRNA to rcDNA and has dominated the clinical HBV antiviral treatments in practice for its convenience and being highly potent. NAs treatment rarely achieves HBV cure (HBsAg clearance or seroconversion by definition). The other category works on the host immune system and tries to recover the exhausted HBV-specific immune response. Interferon alpha (IFNa) (native form or PEG-modified form with an extended half-life in vivo) are sometimes applied to a small percentage of selected chronic hepatitis B (CHB) patients aiming to achieve HBV functional cure through boosting host immune system. However, HBV clearance, which means the eradication of total HBV cccDNA, or, to a lesser extent, HBV functional cure, which means persistently HBV suppression when withdrawal of medical interference, is still an unmet goal for most of the CHB patients, because HBV replication template, covalently closed circular DNA (cccDNA), is not directly targeted and eliminated by current therapeutics [4]. In the history of combating virus infection, the achievement of hepatitis C virus (HCV) therapeutic direct-acting antiviral agents (DAA) served as a typical case for successful drug development, which took only 20 years from the first identification of HCV in 1989 to pilot DAA clinical trials in the early

2010s [5, 6]. Successful HCV replicating cell culture models (Huh-7.5 cells, etc.) contributed tremendously to drug screening and viral replication and greatly expedited the movement of drug pipeline [7–9]. However, as for HBV drug development, the shortage and the slow progress of cellular and animal models for culturing HBV virus significantly impeded the advance of the novel HBV antiviral therapy. Until now, HBV NA inhibitors, as known, were borrowed from other virus, such as HSV (entecavir) [10] and HIV (lamivudine, adefovir, and tenofovir) [11, 12], due to the shared evolutionally conserved structure in their reverse transcriptase domain of polymerase between HBV and HIV [13]. Lamivudine, adefovir, and tenofovir were firstly screened and tested for their inhibitory capability against HIV virus in HIV cell infection models [11, 12]. HBV cccDNA, the template for producing progeny virus, which exists in the form of highly organized minichromosome with histone and epigenetics proteins, is speculated to be extremely similar to the human chromosome in structure and stability and thereby can persist up to several decades in HBV-infected individuals [4, 14, 15]. HBV functional cure (elimination) and HBV clearance (eradication) require the permanently silencing or erasing cccDNA, respectively. Therefore, novel strategies targeting cccDNA are the top priority for HBV treatment.

HBV has a unique genome. The 3.2 kb genome contains four overlapping and frameshifted open reading frames in a highly compacted and economically organized manner, and, importantly, all genome sequence is utilized to encode seven viral proteins, including HBeAg, HBcAg, HBx, Pol, large surface (L), middle surface (M), and small surface (S), with all promoters and their regulatory elements embedded in the coding sequence region. In particular, HBV accomplishes its full life cycle through the generation of a 3.5 kb pregenomic RNA (pgRNA) intermediate, which has duplicated 5′ and 3′ sequence that plays a critical role for polymerase jumping during reverse transcription into rcDNA. Therefore, only plasmids with overlength genome (usually 2-fold, 1.4-fold, 1.3-fold, 1.2-fold, etc.) which contains HBV core promoter in the overlength region for generating full-length HBV pgRNA are employed to produce mature HBV viral particles in cell culture models and in HBV DNA transfection mouse models. Fortunately, various HBV cell culture and mouse models were generated and gradually improved for investigating HBV biology and pathogenesis and for translational research. The progression is summarized in the following sections.

2 HBV Cell Culture Models

2.1 HBV DNA Integrated Stable Cell Models

HepG2 cell, derived from a liver hepatocellular carcinoma of a 15-year-old Caucasian male, and Huh7 cell, originally taken from a liver tumor of a 57-year-old Japanese male, are often used in hepatitis virus study. HepG2 cell is often preferred

for HBV study, while Huh7 cell is often applied to HCV study. HBV can fulfill the whole life cycle and generate cccDNA and produce HBV virus particles in HepG2 cells transfected with genome overlength HBV plasmid [16]. However, HepG2 was difficult to be transfected especially in the 1980s to early 2000s due to the absence of high-performance DNA transfection reagents, resulting in too low titers of HBV virus. Moreover, a huge amount of input DNA severely interfered with the accurate and quantitative measurement of HBV cccDNA formation and HBV replication, making data analysis troublesome and challenging when evaluating the effectiveness of antiviral against HBV [17]. Correspondingly, to circumvent plasmid transfection procedure, HepG2.2.15 cell line was generated through the integration of a tandem of twofold HBV genome DNA fragment into HepG2 cells in 1987 [18] and has contributed significantly to understanding HBV life cycle and viral-host interaction and to assessing HBV antiviral ever since [19]. In HepG2.2.15 cells, the integrated HBV genome keeps generating HBV pgRNA and constitutively producing viral particles, resulting in an excessive amount of intracellular rcDNA which still significantly, but to a lesser extent compared to HBV plasmid transfection, intervene the detection of cccDNA in Southern blot and PCR detection.

Next, with an improvement, the second generation of HBV producing stable cell line, HepAD38, containing a Tet-Off inducible controlled HBV production, was reported in 1997 [20]. HBV genome was inserted downstream a CMV test promoter and ahead of a polyA signal sequence derived from preproinsulin gene 2 (pp-ins2) in HepG2 cell genome. In the absence of tetracycline, CMV test promoter is open and actively generates pgRNA which provides enough templates for subsequent synthesis of a large amount of HBV rcDNA. HBV cccDNA also formed and accumulated from nucleus translocated rcDNA through internal cycling. In contrast, in the presence of tetracycline, CMV test promoter is shut down (HBsAg is robustly produced), preventing new HBV pgRNA generation, and its subsequent new rcDNA conversion, resulting in gradual consumption of stock rcDNA by continuously releasing viral particle, and finally HBV cccDNA is brought out to an easily monitored and measured level. The HepAD38 cell has now been widely used in many laboratories. Based on a similar rationale, an improved HepDE19 cell line was established in 2012 [21]. In the cell line, integrated HBV genome produced full-length PreCore RNA with a mutated ATG start code, abolishing HBeAg production from integrated HBV DNA sequence. Secreted HBeAg can only be generated from the newly formed HBV cccDNA and therefore provide a reliable cccDNA-dependent surrogate. However, because HBeAg and HBcAg are highly homologues in protein sequence, complete differentiation of HBeAg from HBcAg cannot be obtained by common HBeAg ELISA reagents, resulting in unreliable measurement of a trace amount of HBeAg. To make the system more suitable for high-throughput drug screening, a Flag epitope tag was introduced in the advanced version based on HepDE19 cell line [22]. In this new cell line, HepBHAe82, Flag epitope was placed in-frame between the mutated PreCore ATG codon and Core ATG and didn't comprise the generation of pgRNA and the subsequent reverse transcription and cccDNA formation. And in order to get an elevated level of cccDNA, two tandem stop codons were introduced into S gene, totally terminating the surface protein production,

which shut down the generation of the mature virus and forced all packaged rcDNA into internal recycling pathway to form cccDNA. As commercial Flag antibody is highly specific and sensitive, Flag detection can be easily carried out conveniently. Therefore, the flag tag incorporated in HBeAg serve as a simple, specific, and sensitive cccDNA surrogate, and high-throughput drug selection against cccDNA formation and stability becomes workable and affordable.

The above cell culture models are very valuable in dissecting HBV viral life cycle from RNA transcription and transportation, protein translation, reverse transcription, internal cccDNA recycling, and viral assembly and secretion and in analyzing viral-host interaction such as escape of host detection and subversion of host factor restriction and also provide a reliable and readily available virus resource. However, the early stage of the HBV life cycle is missing in the above system including from virus entry, rcDNA decapitation and nucleus translocation, to cccDNA formation and minichromosome establishment.

2.2 HBV Infection Cell Models

Primary human hepatocytes [23] and tree shrew hepatocytes [24] are natural cells permissive for in vitro HBV infection; nevertheless, their unavailability and high cost restrict their broad application in basic scientific researches. The characterization of the HepaRG cell in 2002, the first bipotent cell line could be differentiated into the hepatic-like or biliary-like cells in the laboratory and allowed HBV infection, was a breakthrough for HBV study [25, 26]. HepaRG cells can maintain their proliferation status and keep passages in normal medium and can be polarized and differentiated into hepatic-like cells in DMSO conditional medium. Differentiated HepaRG cells are long-lived and less proliferative, a status morphologically and functionally similar to primary human hepatocytes [27]. After HBV infection in differentiated HepaRG cells, cccDNA was formed and produced high-level HBV continuously for more than 3 weeks. Nonetheless, one major obstacle accompanied with the HepaRG infection system is that the procedure of differentiation of HepaRG cells usually took a first 2-week plantation and another 2-week DMSO differentiation with timing medium changes before initiation of HBV infection, which is challenging and labor-intensive. Meanwhile, for successful HepaRG cell differentiation, all cell culture serum and special ingredients require to be ordered from a special French provider.

In 2012, the identification of HBV viral receptor (sodium-taurocholate cotransporting polypeptide NTCP, also known as SLC10A1, a cotransporter participating in the enterohepatic circulation of bile acids) was a milestone achievement in the field [28]. The establishment of HepG2-NTCP cell line that permitted HBV entry independent of a special cell line opened a new era for HBV study [28]. Subsequent studies confirmed that overexpression of human NTCP in HepG2, Huh7, and HepaRG cells empowered their susceptibility to HBV infection [29, 30]. Compared with HepaRG cells, NTCP overexpressing cells require neither long pretreatment

before HBV infection nor special ingredients, significantly simplifying the cell differentiation step and reducing the cost, and have been warmly welcomed by the fields. It only takes 1-day cell plantation and another day DMSO treatment before commencing HBV infection.

Both HepaRG HBV infection model and human NTCP overexpression cells have greatly promoted the understanding of HBV infection and replication. However, in both infection systems, successful HBV infection stringently requires a high concentration of HBV viral particles (preferably >1000 genome copies/cell) with the aid of PEG8000, and HBV infection is a transient infection, and second round of HBV infection rarely occurs, suggesting another co-receptor(s) is still necessary for establishing efficient infection. In addition, the production of enough high titers of HBV virus for infection is also labor extensive, which needs to culture large volume of cells and two rounds of concentration. In this chapter, we will describe a detailed protocol about HBV infection in HepG2-NTCP cells.

2.3 HBV Recombinant cccDNA Transfection Cell Models

Strategies targeting cccDNA ranks the top priority in achieving HBV cure in combating HBV. In an alternative approach to having cccDNA in cells, several groups managed to generate recombinant HBV cccDNA (rc-cccDNA), delivery of which into HepG2 cell achieved persistent HBV production, circumventing live HBV infection [31–34]. Onefold HBV genome was first linearized and inserted into a plasmid between some cis-elements accounting for plasmid recombination. The HBV plasmids could be amplified simply with commonly in bacteria strains using standard plasmid preparation protocol. Then, HBV rc-cccDNA was generated in two different ways. In one way, the purified HBV plasmid was transfected into HepG2 cells, where it was converted into a minicircle HBV cccDNA through either a Cre-/LoxP-mediated recombination [34]. The intracellular recombination efficiency was not enough to process the overfed amount of plasmid transfected into cells, leaving too much parental HBV plasmid untouched. In the other way, in a specially engineered bacteria strain to express inducible PhiC31 integrase and Sce I endonuclease, rc-cccDNA was obtained through PhiC31 integrase-mediated attP/attB recombination, and the plasmid backbone and residual un-recombinated parental plasmid were degraded by Sce I [35]. Therefore, high-purity HBV rc-cccDNA can be obtained and directly transfected into cells [31–33].

HBV rc-cccDNA exhibited improved stability and formed minichromosome, one major characteristic extremely inherent to native HBV cccDNA [31–34]. Another advantage of the rc-cccDNA transfection system is that preparation of infectious HBV particles becomes unnecessary, and thereby HBV rc-cccDNA can be genetically manipulated to be replication defective, excluding rcDNA generation and their contamination, making it possible to study the cccDNA biology in a clear background. The region selecting is also flexible. HBV Pol, Core, and PreC/C genes were reported to be compatible to insert in rc-cccDNA [31–34]. After recombination,

a "lesion" residual marker will be kept in rc-cccDNA. To avoid its blockage of viral protein production, intron sequences (splicing donor and receptor) were placed closely flanking the "lesion" sequence, enabling the intron-lesion-intron to be spliced off during mRNA maturation processing steps. Furthermore, in our version of rc-cccDNA, we introduced a reporter gene Gaussia luciferase (GLuc) into HBV core region, the secreted GLuc in culture supernatant served as a simple surrogate to predict the quantity and activity of the intracellular rc-cccDNA [32]. The system is valuable for screening and evaluating antivirals directly targeting cccDNA. We will entail our cccDNA protocols in this chapter.

3 HBV Animal Models

HBV, known to be a strict hepatic tropic virus, has a very limited host spectrum. Only chimpanzee [36], Mauritian cynomolgus monkey [37], treeshrew (*Tupaia belangeri*) [38, 39], and woodchuck [40] have been reported to be naturally permissive for HBV infection and thereby are exploited to investigate viral-host interaction and pathogenesis including acute hepatitis, chronic hepatitis, fibrosis, cirrhosis, and HCC. Meanwhile, other evolutionally relevant and representative members of hepadnaviridae, such as duck HBV and woodchuck HBV (WHV), were also applied to study HBV infection and contributed significantly to the understanding of HBV replication and its pathogenesis in their own hosts [41–44]. However, the above models are not widely engaged because of the shortage of reagents for detecting and measuring host factors and for monitoring host immune response, because of the expensive facilities required for animal maintenance and care, and because of ethics restriction. Most of the attempts returned to the generation of HBV models based on housed laboratory mouse strains which have well-characterized genetic backgrounds and are easy to be manipulated for special purposes. And also, research reagents and type of equipment for the mouse access easily. From a historical perspective, HBV mouse models have evolved and greatly improved after several generations, including from original HBV DNA transgenic to HBV DNA transfection and finally to HBV infection in the liver chimeric mouse. The history of mouse HBV models is summarized as the following.

3.1 HBV Transgenic Mouse Model

HBV transgenic mice were first generated through introducing a 1.3-fold HBV genome into the mouse genome in the 1990s [45]. Similar to HepG2.2.15 cells, the integrated HBV genome supports all HBV RNAs and proteins generation in mouse hepatocytes, and HBV virus can accomplish the late stage of life cycle including rcDNA synthesis from pgRNA, viral assembly, and mature virus secretion. The model has been used to analyze host factor that can suppress HBV production

in vivo and to evaluate the HBV antivirals [46, 47]. Unfortunately, the model has setbacks including undetectable HBV cccDNA in mouse hepatocytes and natural immune tolerance to HBV antigens due to the integrated HBV genome in the host chromosome, resulting in the absence of HBV-related liver diseases.

3.2 HBV In Vivo Transfection Mouse Models

3.2.1 HBV Plasmid DNA Hydrodynamic Injection Models

HBV hydrodynamic injection mouse model was one major breakthrough and firstly developed to fulfill the requirement of nonintegrated HBV genome in the mouse in 2002 [48]. In principle, hydrodynamic injection of a 10% mouse weight (g) (v/w) volume of HBV plasmid into mouse tail vein in an only several seconds caused a transiently high blood pressure, which mechanically squeezed HBV DNA into mouse hepatocyte, and led to persistent HBV production [48]. The in vivo transfection model successfully avoided HBV integration. Accordingly, the injected mice possess the mature immune system which recognized HBV antigens and initiated viral specific immune response when HBV plasmid was injected [49, 50]. The new model system can be used to test the HBV cure strategy and to study HBV immunology [51, 52]. Nevertheless, the hydrodynamic injection model possesses the following disadvantages. Firstly, the non-HBV immune response is elicited for the existence of the plasmid backbone with a bacterial origin, which usually leads to the silence and elimination of the whole HBV plasmid. In an improved version, Adeno-associated virus (AAV) element was inserted into the plasmid to extend HBV DNA maintenance, but with limited success, in mouse hepatocytes [49]. Secondly, this HBV DNA model picks mice, and only mice with C3H background, with a lower level of interferon generation, are hospitable to plasmid backbone DNA and are often selected for HBV plasmid injection study [51]. Thirdly, hydrodynamic injection is a technically challenging procedure, requiring well-experienced operators. Finally, the intrahepatic transfection efficiency in mouse liver was only about 5 percent, a pretty low level compared with real HBV infection.

3.2.2 Viral Vector-Mediated HBV Genome DNA Delivery Models

To increase the in vivo transfection efficiency and reduce the non-HBV-related immune response, hepatic tropic viral vectors including Adenovirus [53], Baculovirus [54], and Adeno-associated virus (AAV) [55, 56] were utilized to deliver HBV 1.2–1.3-fold genome into the mouse liver. Both improved HBV stability and elevated transfection efficiency were achieved. Because Adenovirus and Baculovirus vectors have the very large genomes and encode numerous non-HBV

viral products which are potentially immunogenic and elicit a strong non-HBV-related immune response, interpretation of HBV-related immune response and pathogenesis is technically challenging. In contrast, AAV-HBV vector has minimal AAV genome with only essential AAV ITR sequence, which is responsible for viral packaged and doesn't encode any AAV viral proteins. Therefore, AAV-HBV model keeps a clean background when HBV specific immune response is analyzed. AAV-HBV mouse model can persistently produce HBV virus particles and HBsAg for more than 1 year [55, 56] and has been widely used. But no HBV induced liver pathogenesis was observed in the AAV-HBV model.

One major common disadvantage of HBV transgenic mice, HBV DNA hydrodynamic injection mice, Adenovirus HBV mice, and Baculovirus HBV mice, is that HBV cccDNA was rarely and not convincingly detected, suggesting that HBV cccDNA intracellular recycling pathway is severely impaired in mouse hepatocytes. Interestingly, only HBV cccDNA was recently observed in AAV-HBV mouse [57]. One possible explanation was that cccDNA was generated through recombination during the process of AAV-HBV single-strand DNA (ssDNA) conversion to dsDNA in mouse hepatocytes but not from rcDNA through intracellular recycling pathway.

3.2.3 HBV rc-cccDNA Mouse Models

One breakthrough was recorded that recombinant cccDNA was firstly generated in mouse hepatocytes from hydrodynamic injected HBV plasmid through Cre-/Loxp-mediated recombination in a pilot study in 2014 [34]. Hydrodynamic co-delivery of a plasmid expressing Cre recombinase and a plasmid containing double-LoxP flanked HBV DNA sequence into mouse hepatocytes successfully generated HBV rc-cccDNA, which, as the template, performs a similar function to real HBV cccDNA in producing mature HBV virus. The recombination took place in mouse hepatocytes, and plasmid backbone after excision was still kept in the mouse liver and still evoked host immune response, which reduced the rc-cccDNA stability and persistence in vivo. In the model, cccDNA only lasted for several weeks. To overcome the shortcoming, an improved version of cccDNA mouse was developed by the same group recently through Adenovirus delivering linear HBV genome into a Cre transgenic mouse [58]. Attractively, persistent HBV production resulted in advanced liver pathogenesis, which was rarely achieved in all mouse models previously mentioned. The study indicated that real cccDNA is required for HBV to cause liver damage and the subsequent fibrosis in mice. Alternatively, hydrodynamic injected recombinant minicircle cccDNA prepared from bacteria also achieved long-term maintenance in C3H mice, a novel and simple approach independent of transgenic mice for investigating HBV cccDNA-related biology and for evaluating anti-HBV drugs [33].

3.3 HBV Infection in Liver Humanized Mouse Models

Ideally, a mouse model should support HBV whole life cycle starting from virus attachment and entry, rcDNA decapitation and translocation into the nucleus, subsequent cccDNA generation, minichromosome formation, viral gene expression, and finally to virus assembly and secretion. The first identification of human NTCP (huNTCP) as HBV entry receptor in hepatocytes evoked great enthusiasm for the whole field to generate human NTCP transgenic mouse, expecting that the huNTCP transgenic mouse will be permissive for proliferative HBV infection [28]. Surprisingly, huNTCP transgenic mice fell through the original anticipations and failed to support productive HBV infection [59]. Then, mouse immune system was thought to as a key player to effectively control virus replication even if HBV infection succeeded in the huNTCP transgenic mice. Recently, in the immune deficient settings (NRG mouse with no T, B, and NK cells), overexpressing huNTCP allowed HBV infection but still failed to support HBV replication by measuring HBV DNA, HBsAg viral products, providing convincing data that another element(s) is playing critical roles in restricting HBV from forming functional cccDNA even after successful entry step [60].

Currently, only the liver humanized mouse can support robust HBV infection. A landmark breakthrough in the history of the liver humanized mouse was the generation of uPA/Hep chimeric liver mouse in 2001 [61]. In the immune-deficient mice, hepatic-specific expressing urokinase-type plasminogen activator (uPA) induced hepatocyte loss spontaneously, which provided spare niches allowing transplanted human hepatocyte to implantation, growth, and repopulation to high percentage after around 10 weeks. The liver chimeric mice supported a high level of HBV replication [62–64]. As the first real HBV infection model, uPA-based humanized mice with chimeric liver was quickly spread to the whole hepatitis research field, and different types of uPA mice were generated using similar strategies in the mice with different backgrounds [63, 64]. However, constitutive uPA expression in an uncontrolled manner always led to high mortality in newborn pups if the human hepatocytes were not timely and properly transplanted, resulting in a poor breeding efficiency and a narrow time windows for transplantation [65]. Thus, the demanding and costly colony maintenance restricted the wide application of this model.

The next milestone in the field was the generation of drug controlled mouse hepatocyte damage model in Fah-knockout mice in 2007 [66]. The mouse Fumarylacetoacetate hydrolase (Fah) gene encodes the last enzyme in the tyrosine catabolism pathway and is hepatic-specific active in mouse hepatocytes. Knockout of Fah diverted the metabolic pathway to the hepatic accumulation of toxic tyrosine metabolic intermediates, which caused the subsequent death of mouse hepatocytes. The chemical 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a clinically approved drug to treat hereditary tyrosinemia type 1, can block the first step of tyrosine metabolism. Adjustment of the NTBC concentration can fine manipulate the accumulation of toxic intermediates, generating a tunable hepatocyte injury for stepwise human hepatocytes repopulation [66, 67]. Another important

virtue is that NTBC drug can be passed from mother to pups through milk, greatly reducing pup loss and simplifying the colony maintenance. Fah-knockout mice are very popular in many labs.

An important issue about mouse background should be pointed out here as of their application for human cells repopulation. In the immune deficient status without T, B, and NK cells, macrophage cells become the major force to face new exotic "invader" and recognize exotic cells through its antenna molecular Sirpa, which justify a killing signal or not when coupled with its ligand molecular CD49 on the transplanted cells. Immune deficient mice (no T, B, and NK cells) with Balb/c and C57 background are not hospitality to transplanted human cells due to the mismatch of mouse Sirpa with human CD49 and initiate a slow killing program against transplanted human cells. Fortuitously, the Sirpa protein of NOD background shares higher similar to human Sirpa proteins, and a small number of human hematopoietic stem cells can achieve a high level of human cell repopulation [68]. For the first time, we generated NRG/Fah−/− immune deficient mouse through directly editing NRG embryos (NOD background) with CRISPR/Cas9 technology in our lab [69]. We have optimized the NTBC drug protocol and gotten human hepatocytes repopulated chimeric mouse liver, which support HBV replication. In this chapter, we will describe a protocol in detail about how to achieve increasing degrees of liver damage by supplying water with reduced level of NTBC, such as 100%, 25%, 12%, 6%, and 0%, and about how to repopulated to repopulate of NRG/Fah−/− mice with small number of human hepatocytes for HBV infection study. Our protocol will also be applicable to Fah−/− mice with other backgrounds.

TK-NOG mice is another drug-inducible liver damage model, which was also generated in NOG immune deficient mouse through the hepatic-specific expression of Herpes simplex virus type 1thymidine kinase (HSVtk) mouse in 2011 [70]. Brief exposure of TK-NOG to a nontoxic dose of pre-drug ganciclovir (GCV) resulted in gradual ablation of mouse hepatocytes and releasing space for human hepatocyte engraftment and maintenance. TK-NOG mice repopulated with human hepatocytes supported robust HBV replication and were widely used in HBV drugs screening and evaluation [71, 72]. Similar to all other transgenic mice with HSVtk gene, the minor defect of TK-NOG was the low breeding efficiency caused by male sterility, and thereby the colony maintenance requires mating female TK-NOG mice with wild type male NOG mice and genotyping the transgenic offsprings [70].

4 Protocol 1. HepG2-NTCP HBV Infection Model

4.1 Materials

Cell

HepG2-hNTCP-C4, engineered from human hepatocellular carcinoma cell line HepG2, constitutively expressing human NTCP, a gift from Dr. Takaji Wakita (Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan).

Reagents

1. DEME (Gibco, 11,995,065).
2. DMEM complete medium. Including DMEM with 10% FBS, 10 mM HEPES, 1x MEM NEAA, 1 x GlutaMAX-I, 5 μg/ml insulin, and 50 μM hydrocortisone.
3. DMEM infection medium. Including DMEM, 1 mM HEPES, 1 x MEM NEAA, 1 x GlutaMAX-I, 5 μg/ml insulin, and 50 μM hydrocortisone.
4. 1 x DPBS (Gibco, 14,190,144).
5. 1 M HEPES (Gibco, 15,630,080).
6. 100 x MEM NEAA (Gibco, 11,140,050).
7. 100 x GlutaMAX-I (Gibco, 35,050,061).
8. Insulin solution human (Sigma, I9278).
9. Hydrocortisone 21-hemisuccinate sodium salt (Sigma, H2270).
10. 100 mMHydrocortisone solution. Dissolving 100 mg Hydrocortisone powder into2.06 ml ddH_2O, filtered through 0.22 μmmillipore syringe filter, aliquotand store at −20 °C.
11. G418 sulfate (Invivogene, ant-gn-1).
12. Doxycycline (Sigma, D9891).
13. PEG 8000 (Sigma, P2139).
14. 40% PEG 8000 solution. Weighting 100 g PEG 8000 powder and dissolving into 1 x DPBS to make a final volume is 250 ml. When the solution becomes completely clear, filter the solution with 0.22 μm bottle-top vacuum filter. Store at RT or 4 °C for long-term.
15. SYBR green ROX mix (Thermo Scientific, 1163A).
16. DMSO (Sigma, D4540).
17. 0.25% Trypsin (Gibco, 15,050,065).

Supplies

1. QIAampMinElute Virus Spin Kit (Qiagen, 57,704)
2. 0.45 μmbottle-top vacuum filter (Corning, 430,768)
3. 0.22 μmbottle-top vacuum filter (Corning, 430,767)
4. Counting slides, dual chamber (BioRad, 145–0011)
5. Millex-GP Syringe Filter Unit, 0.22 μm (Millipore, SLGP033RS)

4.2 Methods

1. **HBV virus collection and concentration**

   (1) Seed 3 million HepAD38 cells in a T75 flask with DMEM complete medium and culture in a 37 °C CO_2 incubator. HepAD38 cells are grown in the presence of 1 μg/ml doxycycline and 500 μg/ml G418 until they get 80% confluence.
   (2) Change to the medium without doxycycline and G418.
   (3) Keep culturing HepAD38 for another 10–14 days and collect the supernatant every 2 days. Store the supernatant at 4 °C.

(4) After the final collection, put all the supernatant together and centrifuge at 250 g at 4 °C for 20 min.
(5) Keep the supernatant and discard the cell debris.
(6) Clarify the supernatant by sterile filtering through a 0.45 μmbottle-top vacuum filter.
(7) Add 40% PEG 8000 solution to the supernatant to an 8% final concentration.
(8) Completely mix container by inverting at least 30 times and incubate at 4 °C overnight.
(9) Centrifuge at 10,000 × g for 1 h with a fixed angle rotor at 4 °C.
(10) Remove the supernatant and use 1/100 of the original volume of DEME to suspend the pellet.
(11) Shake or pipetting the virus suspension gently at 4 °C for a couple of hours.
(12) Aliquot HBV virus and store at −80 °C ready to use.

2. **HBV virus titer detection**

(1) Take 20 to 50 μl concentrated HBV virus, follow the manufacturer's protocol of QIAampMinElute Virus Spin Kit, and elute HBV extracted DNA via 40 μl buffer AVE (see Note 1).
(2) Perform real-time qPCR assay to measure HBV quantification. Put 5 μl HBV DNA into a 15 μl reaction system as a template.
(3) Per reaction, the system is 7.5 μl SYBR green ROX mix, 1 μl 10 μM primer mixture (HBV-total-F and HBV-total-R), 1.5 μl ddH2O, and 5 μl extracted HBV DNA. The detailed primers' information is listed below, HBV-total-F primer (5′-3′): GTTGCCCGTTTGTCCTCTAATTC; HBV-total-R primer (5′–3′): GGAGGGATACATAGAGGTTCCTTGA.
(4) The reaction mixture was denatured at 95 °C for 10 min, followed by 40 cycles of 95 °C denaturation for 30 s, and 60 °C annealing for 30 s and 72 °C elongation for 30 s. The level of HBV DNA was quantified on a QuantStudio™ 6 Flex Real-Time PCR System instrument (see Note 2, 3).

3. **HBV infection of HepG2-hNTCP-C4 cells**

Day 0

(1) Seed HepG2-hNTCP-C4 cells in a 48-well plate at a density of 70,000 cells per well in 250 μl DMEM complete medium.

(2) 3–5 h later, when most of the cells adhere to the wells, change to 250 μl DMEM complete medium containing 2–3% DMSO per well (see Note 4).

Day 1

(1) Using trypsin to digest HepG2-hNTCP-C4 cells from one well and account total cell numbers via counting slides through TC20 automated cell counter (BioRad).
(2) Set conditions: 500 MOI (Multiplicity of infection). Based on total cell numbers per well, calculate how much viruses needed. According to virus titer, calculate total viral volume.

(3) Before infection, wash cells with DMEM infection medium three times.
(4) Set three groups, mock (uninfected), infection and infection plus reverse transcriptase inhibitor, and tenofovir (TFV).
(5) For the mock group, add 200 μl DMEM infection medium containing 2–3% DMSO and 4% PEG 8000 per well; for infection and infection plus TFV groups, add 200 μl DMEM infection medium containing 2–3% DMSO, 4% PEG 8000 and individual HBV virus per well.
(6) Mix well and culture the whole plate in a 37 °C CO2 incubator overnight (see Note 5).

Day 2

(1) After 16–18 h of infection, wash the cells with pre-warmed DEME or 1X DPBS five times.
(2) Add 250 μl DMEM complete medium containing 2–3% DMSO per well for mock and infection groups and keep the whole plate in a 37 °C CO2 incubator.
(3) In infection plus TFV group, add 250 μl DMEM complete medium containing 2–3% DMSO and 50 μM TFV per well until the end day of the experiment and keep the whole plate in a 37 °C CO2 incubator.
(4) Collect supernatant 3, 6, 9, 13, 17, and 21 days after infection and store all the samples at −20 °C until to be measured.

4. **Analyze HBV replication kinetics through real-time qPCR assay**

   (1) Take 200 μl per sample and extract HBV DNA based on the protocol of QIAampMinElute Virus Spin Kit and elute HBV extracted DNA via 40 μl buffer AVE.
   (2) Run real-time qPCR to quantify HBV DNA. The method is totally the same as that in "HBV virus titer detection" part (see Note 6) (Fig. 5.1).

Notes

1. Usually, the viral titer is very high from HepAD38 cells if the cell condition is good. Based on our experience, after concentrating 300 ml supernatant to 300 μl viral inoculum, the titer can achieve 10^10 copies per ml. Therefore, take only 20–50 μl concentrated HBV virus and add 150–180 μl DPBS to make a 200 μl sample (suggested by the kit protocol) for HBV DNA extraction enough.
2. For the real-time qPCR program, using 60 °C for annealing and elongation is still fine under the same reaction and via the same machine.
3. Use a plasmid containing HBV whole genome or amplified region as the standard to quantify HBV viral titer. Converting the concentration of plasmid to copies per ml and according to Ct value to draw a standard curve and based on the Ct value of HBV DNA sample, calculate virus titer.
4. Once most of the cells can adhere to the plate, add DMSO to the culture medium. DMSO is toxic to cells under higher concentration, but it is also needed to induce HepG2-hNTCP-C4 to be accessible to HBV infection. Less than 4% of DMSO can be acceptable.

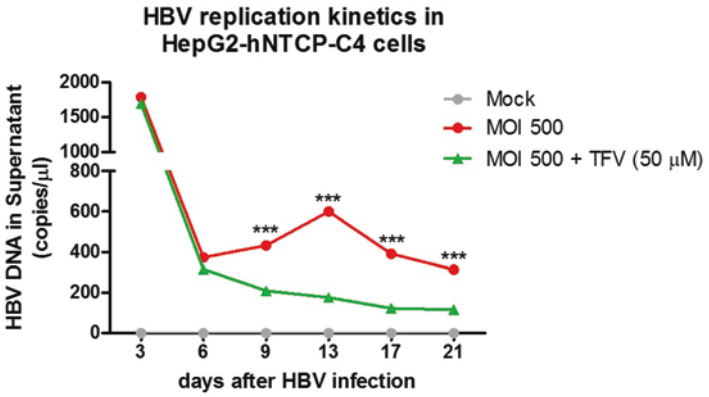

Fig. 5.1 HBV DNA replication kinetics in HepG2-hNTCP-C4 cells. HepG2-hNTCP-C4 cells were infected by HBV (concentrated from the supernatant of HepAD38 cells) at a MOI 500 with or without TFV (working concentration is 50 μM) in the presence of 2–3% DMSO and 4% PEG 8000. Supernatant was collected 3, 6, 9, 13, 17, and 21 days after infection and HBV DNA quantity analyzed via real-time qPCR method. ***, $P < 0.001$. Statistical analysis was performed by student's t test with GraphPad Prism 5 software. Data are presented as means ± SEM

5. The HBV virus incubation time should be around 16–18 h. Do not exceed 24 h; otherwise, most of the cells will die.
6. The HBV replication kinetics in HepG2-hNTCP-C4 supernatant should decrease first and increase later in a successful infection, and TFV, if added, will totally block the virus replication and prevent virus releasement (Fig. 5.1).

5 Protocol 2. HBV rc-cccDNA Transfection Model

5.1 Materials

Cell

HepG2 ATCC® Catalogue Number: HB-8065.

Reagents

1. DMEM (Gibco, 11995065)
2. 1 x DPBS (Gibco, 14190144)
3. 1 M HEPES (Gibco, 15630080)
4. 100 x MEM NEAA (Gibco, 11140050)
5. 100 x GlutaMAX-I (Gibco, 35050061)
6. DMEM complete medium, DMEM with 10% FBS, 10 mM HEPES, 1x MEM NEAA, 1 x GlutaMAX-I
7. 0.25% trypsin (Gibco, 15050065)
8. Opti-MEM I Reduced-Serum Medium (Gibco, 319850070)
9. Lipofectamine 3000 Transfection Reagent (Invitrogen, L3000015)

10. Recombinant HBV cccDNA (rc-cccDNA), homemade in the lab
11. Renilla-Glo® Luciferase Assay System (Promega, E2710)

Supplies

1. QIAamp MinElute Virus Spin Kit (Qiagen, 57704)
2. TC20™ Automated Cell Counter (BioRad, 1450102)
3. Counting slides, dual chamber (BioRad, 145–0011)
4. Centrifuge (Eppendorf, 5810R)
5. GloMax® 96 Microplate Luminometer (Promega)
6. 10 cm cell culture plate (Corning)

5.2 Methods

5.2.1 Recombinant HBV cccDNA transfection

Step 1. Prepare single cells for transfection.

(1) Confluent HepG2 cells are washed once with 5 ml 1X DPBS and treated with 1 ml 0.25% trypsin at 37 °C until cell detached (see Note 1).
(2) Add 5 ml complete DMEM medium, make single cell suspended through several pipetting, and transfer cells into a 15 ml falcon tube.
(3) Spin down cells at 1500 rpm for 5 min.
(4) Resuspend cell pellet with Opti-MEM I, and take 20ul to calculate cell number using a cell counter (see Note2).
(5) Aliquot cells for 10–15 million/tube (see Note 3).

Step 2. Prepare rc-cccDNA transfection solution.

(1) Prepare the DNA mixture. 15 ml tube 1: add 5 ml OptiMEM-I, 9.5ug pCDNA3 stuff DNA, 0.5ug cccDNA, + 20 ul P3000.
(2) Prepare the liposome solution. 15 ml tube 2: add 5 ml OptiMEM-I, 20ul Lipofectamine3000 (see Note 4).
(3) Vortex gently for 30 s. Place at RT for 5 min. Then mix tube 1 and tube 2 and place at RT until use.

Step 3. Cell transfection and culture.

(1) Spin down aliquot cells at 1500 rpm for 5min at RT.
(2) Resuspend 10–15 M cells with 10 m rc-cccDNA mixture and plate cells in one 10 cm culture dish. Put back into cell incubator at 37 °C.
(3) 20–24 h post-transfection, replace OptiMEM I with 10 ml complete DMEM medium and keep culture in 37 °C (see Note 5).

5.2.2 HBV cccDNA detection

(1) At day 3 post-transfection, keep the culture supernatant. Wash cell with 5 ml 1X DPBS, add 1 ml 0.25% trypsin, and put back to 37 °C incubating until cells detached.
(2) Add 5 ml complete DMEM culture medium to make the single cell suspension. Spin down cells at 1500 rpm for 5 min at RT, keeping cell pellet (see Note 6).
(3) Suspend cell pellet with 10 ml complete DMEM culture medium; calculate cell number with cell counter.
(4) Adjust cell number to be 1 million cells/ml, and plate 100 ul cell suspension for each well in 96-well plate.
(5) Change medium every 2 days, and keep cell culture supernatant at −20 °C until detection.
(6) To detect Gaussia Luciferase (Gluc) activity with GloMax® 96 Microplate Luminometer following the instruction, 50 ul was used for detection by adding 50 ul substrate (see Note 7).

Notes

1. Usually, T75 flask is used to culture cell, cell number to be 15–25 million for HepG2 cells.
2. Cell number can be calculated by hemocytometer manually using a microscope.
3. The cell number is calculated for one 10 cm dish. The cell numbers can be scaled up and down.
4. Naturally, the amount of hepatocellular cccDNA is very low. To maximally mimic real HBV infection, we reduce the input rc-cccDNA by adding stuff DNA in our study. The ratio of rc-cccDNA can be adjusted flexibly based on specific purpose.
5. Because HepG2 cells are hard to be transfected, we use several methods to improve transfection efficiency. Firstly, we use liposome 3000 that is much better than liposome 2000, in particular, for HepG2 cell. Secondly, we applied a protocol for suspending cell transfection to HepG2 cell transfection. This transfection protocol is helpful in our experience especially for hard transfection cells, such as HepG2, Vero, MDCK, etc. Thirdly, HepG2 is very tough; we use trypsin digestion to enhance the permissiveness of HepG2 to liposome transfection. Finally, the transfection incubation time can be extended to over 20 h.
6. Trypsin treatment and cell replating into a new plate can maximally get rid of the cell attached and plated attached DNA and reduce input DNA background when measuring intracellular DNA.
7. Gaussia luciferase is a secreted luciferase; it is unnecessary to lyze cell for detection. Gaussia luciferase can use the substrate of Renilla luciferase, and all Renilla detection reagents from Promega work very well for Gaussia luciferase detection,

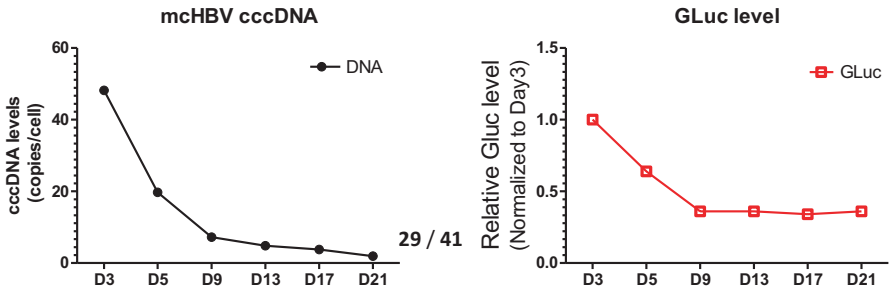

Fig. 5.2 HBV rc-cccDNA persistent in HepG2 cells. One representative experiment is shown

such as Dual-Luciferase® Reporter Assay System, catalogue number:E1910. Despite NEB developing the Gaussia Luciferase system and its detection reagents, BioLux® Gaussia Luciferase Assay Kit (NEB. 3308) is now discontinued (Fig. 5.2).

6 Protocol 3. HBV Infection in NRG-Fah/hu Hep Chimeric Mouse

6.1 Materials

Solution

1. NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (Sigma-Aldrich).
2. Povidone-iodine.
3. Quantitative PCR reagent. Thermo Scientific ABsolute qPCR SYBR green ROX Mix.
4. PCR primers (see Methods).
5. Bethyl Laboratory Human Albumin ELISA Quantitative Set.
6. ELISA coating buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6.
7. ELISA wash solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0.
8. ELISA blocking solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0.
9. Sample/conjugate diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20.
10. Enzyme substrate, TMB Microwell Peroxidase Substrate System (2-C) (KPL, Kirkegaard & Perry Laboratories, Inc.).
11. ELISA stop solution, 1 M H_2SO_4.
12. HBV patient serum (SeraCare Life Science).
13. Freshly made Avertin. Mix 0.25 g of 2,2,2-tribromoethanol, 99% (Alfa Aesar) with 0.5 ml of tert-amyl alcohol (Sigma-Aldrich), and dissolve it in 20 ml of water at 50 °C. Filter-sterilize through 0.2-micron filter.

14. Bleach (10%).
15. ddH20 molecular biology grade (Sigma-Aldrich).
16. 70% ethanol.
17. 1 X DPBS (Gibco).

Equipment

1. Surgery tools. Scissors, curve blunt end forceps, wound clips, wound clip hold, and suture
2. Scalpel
3. Water bath
4. 0.22 μm filter Millex® Syringe Filters (Millipore)
5. Real-time PCR machine
6. 0.5 ml and 1 ml insulin syringe
7. Hair shaver

Methods

1. **Prepare** NTBC drug solution (1000X).

   (1) Dissolve 8 g of NTBC in 1 liter of 0.5% sodium bicarbonate at 65 °C for 30 min. Shake every 3–5 min until all the compound is dissolved (see Note 1).
   (2) Filter the solution using a 0.22um filter. Allocate and store at −20 °C.

2. **Maintenance** of NRG-Fah−/− homozygous mice.

NRG/F homozygous mice must be maintained with 100% NTBC (8 μg/ml). The NTBC drug is pretty stable up to 3 weeks in the cage. Residual water can be filtered and reused when the water bottle is changed.

3. **Hepatocyte** preparation.

   (1) Suspensions of fresh isolated hepatocytes are commercially obtained from Triangle Research Labs (USA). Disinfect the tube surface with 70% ethanol after opening the package.
   (2) Centrifuge cells at 100 g for 10 min.
   (3) Take 5 ml of the supernatant medium to another tube for resuspending the cell pellet in step 5.
   (4) Carefully discard most of supernatant, keeping less than 0.5 ml residual medium.
   (5) Resuspend the cells and transfer to a 1.5 ml Eppendorf tube. Adjust the volume with supernatant medium to 50–70 μl per million cells.
   (6) Count the cells and check cell viability.

4. **Transplantation** with human adult hepatocytes: intrasplenic injection.

Throughout the procedure, standard sterile surgical techniques are used, including bead sterilization of instruments before and in between animals and the use of sterile (prepackaged) gloves. The entire surgery procedure should be performed in a laminar flow hood. It will take 15 min if performed by one person, and it can be

completed in 5–6 min by two people. The mice will wake up in 2–3 h. If bleeding occurs and cannot be stopped, the mouse must be euthanized via CO_2.

(1) Mouse anesthesia. Intraperitoneally inject 500–800 μl of Avertin, depending on the weight of the mouse. It will take 1 to 2 min to completely anesthetize the mouse. Check whether the mouse is fully asleep by squeezing the mouse foot pad.
(2) Shave the left side abdomen around the spleen.
(3) Lay the mouse down on autoclaved paper towel, and sterilize shaved region by sequentially using povidone-iodine and 70% ethanol on the body surface.
(4) Open the skin of the abdomen with a scissor. The incision size is about 1.5–2 cm.
(5) Open the abdomen muscle with a scissor. The incision size is about 0.5–1 cm.
(6) Gently pull out the fat pad under the spleen using a curved blunt-ended forceps.
(7) Slowly inject 1 million hepatocytes in 50–70 μl into the lower pole of the spleen at a speed of about 50 μl in 5 s.
(8) After injection, pull out the needle very slowly to prevent bleeding (see Note 2).
(9) Close the abdominal muscle layer by muscle suture.
(10) Close skin layer by skin staples.
(11) Wound clips will be removed within 10 days after surgery.

5. NTBC **cycling**.

The timing of NTBC cycling is very important, since NTBC controls the level of liver damage (*see* Note 3). The cycle is listed below and summarized in Table 5.1. The remaining NTBC water can be reused at each step. For instance, in each cycle, just add 50 ml of 100% NTBC water to 150 ml autoclaved water to get 25% NTBC water, 96 ml of 25% NTBC water to 104 ml autoclaved water to get 12% NTBC water, and 100 ml of 12% NTBC water to 100 ml autoclaved water to get 6% NTBC water. A lot of NTBC drug will be saved in this manner.

Cycle 1:

Day 0, right after surgery, change water to 25% of NTBC (2 μg/ml).
Day 2, change water bottle to 12% NTBC (0.96 μg/ml).
Day 4, change water bottle to 6% NTBC (0.48 μg/ml).

Table 5.1 Schedule of NTBC drug recycling

	NTBC concentration				
Cycle	25%	12%	6%	0%	100%
1	Day 0	Day 2	Day 4	Day 7–21	Day 21–28
2	Day 28	Day 30	Day 32	Day 35–49	Day 21–28
3	Day 56	Day 58	Day 60	Day 63–77	Day 77–84
4	Day 84	Day 86	Day 88	Day 91–	

Day 7, change water to 0% NTBC.
Day 21, change the water to 100% NTBC.

Cycle 2:

Day 28, change water to 25% of NTBC (2 μg/ml).
Day 30, change water bottle to 12% NTBC (0.96 μg/ml).
Day 32, change water bottle to 6% NTBC (0.48 μg/ml).
Day 35, change water to 0% NTBC.
Day 49, change the water to 100% NTBC.

Cycle 3:

Day 56, change water to 25% of NTBC (2 μg/ml).
Day 58, change water bottle to 12% NTBC (0.96 μg/ml).
Day 60, change water bottle to 6% NTBC (0.48 μg/ml).
Day 63, change water to 0% NTBC.
Day 77, change water to 100% of NTBC (2 μg/ml).

Cycle 4:

Day 84, change water to 25% of NTBC (2 μg/ml).
Day 86, change water bottle to 12% NTBC (0.96 μg/ml).
Day 88, change water bottle to 6% NTBC (0.48 μg/ml).
Day 91, change water to 0% NTBC.

6. Human albumin detection.

A modified protocol from Bethyl Laboratories Human Albumin ELISA Quantitation Set (see Note 5)

(1) Plate coating. Coating antibody dilution 1:1000 (see Note 4). Add 100 μl of diluted coating antibody to each well. Note: Run each standard or sample in duplicate. Incubate at room temperature (20–25 °C) for 1 h. Wash plate five times.
(2) Add 200 μl of blocking solution to each well. Incubate at room temperature for 30 minutes. Wash plate five times.
(3) Dilute the mouse serum sample during step 1 and step 2 100 X during week 3–7 and 1000 X thereafter. Standard dilution: to 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 ng/ml.
(4) Add 100 μl of standard or sample to well. Incubate at room temperature for 1 h. Wash plate five times.
(5) Prepare 1:10,000 dilution of detection antibody dilution (see Note 4). Add 100 μl of diluted HRP detection antibody to each well. Incubate at room temperature for 1 h. Wash plate five times.
(6) Add 100 μl of TMB substrate solution to each well. Develop the plate in the dark at room temperature for 10–15 minutes. Stop reaction by adding 50 μl of stop solution to each well. Measure absorbance on a plate reader at 450 nm.

7. Inoculate NRG/F hu Hep mice with HBV serum.

For infection studies, human serum containing high-titer HBV DNA (>10e9 genome copies/ml) is used. NRG/F hu Hep mice with serum hALB >0.5 mg/ml are infected with HBV virus through the retro-orbit vein, <50 μl/injection.

(1) Anesthetize with Avertin, 500–800 μl intraperitoneally, depending on the weight of the mouse. It will take 1 to 2 min to completely put down the mice. Check the anesthesia effect by squeezing the mouse foot pad.
(2) Lay down the mouse to autoclaved paper towel. Immobilize the mouse head with thumb and point fingers.
(3) Inject the diluted HBV virus (<50 μl) into the retro-orbit. Decontaminate the needle with 10% bleach.

8. Detection of HBV DNA in mouse serum (see Note 5 and Fig. 5.3).

   (1) Serum was collected from tail vein, and HBV viral DNA was extracted with QIAamp® MinElute® Virus Spin Kit according to the instruction. Elute HBV DNA using 50 ul elution buffer.
   (2) The following primers are used to quantify the HBV virus load. Primer 1, HBV2270F GAGTGTGGATTCGCACTCC; Primer 2, HBV2392R GAGGCGAGGGAGTTCTTCT.
   (3) Q-PCR reaction setup. 2 X SYBR buffer 7.5 μl, mix primer (10 μM) 0.105 μl, ddH2O 2.4 μl, and diluted sample 5 μl. Prepare duplicate wells for each sample.
   (4) HBV standard. Use human patient sample with known titer HBV.
   (5) Program for Q-PCR. step 1, 50 °C 2 min; step 2, 95 °C 15 min; step 3, 95 °C 15 s; and step 4: 60 °C 1 min, and go to step 3 for 40 cycles.

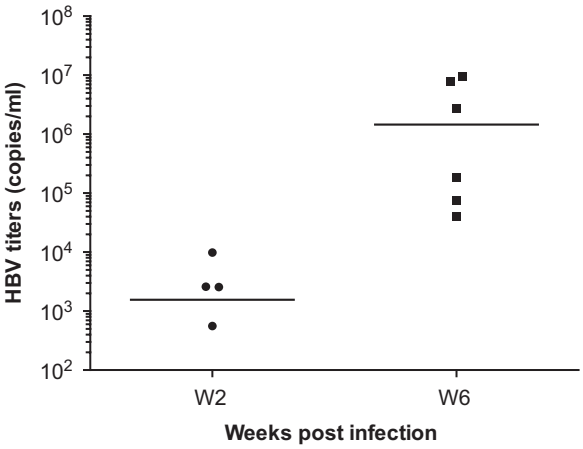

Fig. 5.3 HBV serum DNA in NRG/F hu Hep mouse model

4 Notes

1. The NTBC can be dissolved in 100% DMSO, in which case it does not need to be heated. If dissolved with DMSO, special filters resistant to DMSO should be used.
2. Ligation is required sometimes, but it is not necessary if a small volume of cells is injected and the needle is pulled out very slow. We only use 1 million cells in the NRG/F mice. Up to 5 million cells have been used in published reports. Our experience is that large volume (100 μl) injection with a high concentration of cells usually causes bleeding.
3. If the surgery is not successful, mice will soon die (day 2 to day 10). At around day 21 in the first cycle and day 49 in the second cycle, the NTBC must be put back to 100%. Otherwise, the majority of the transplanted mice will be lost. We have observed a big loss of mice at week 3 and week 7 after surgery if NTBC is not adjusted back to 100%. The time of cycling also relies on the amount and proliferation capacity of human hepatocytes injected. If more cells (> 1 million cells) are use, the interval of NTBC 0% can be extended. If the transfected adult hepatocytes are highly proliferative, the interval of NTBC 0% can be extended. However, this requires consistent checking of the mouse health status.
4. Coating antibody is used at 1:100 dilution in the original manufacturer's protocol. However, 1:100 dilution usually gives a high background. The use of the coating antibody at 1:1000 dilution will not only reduce the background but also greatly reduce the cost. Similarly, the detection antibody is used at 1:10000 dilution instead of 1:7500, as described in manufacture guide.
5. We usually use quantitative PCR to detect the HBV viral genomic DNA. HbsAg ELISA can be used to detect HBsAg.

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Chapter 6
Present and Future Therapies for Chronic Hepatitis B

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Abstract Chronic hepatitis B (CHB) remains the leading cause of liver-related morbidity and mortality across the world. If left untreated, approximately one-third of these patients will progress to severe end-stage liver diseases including liver failure, cirrhosis, and hepatocellular carcinoma (HCC). High level of serum HBV DNA is strongly associated with the development of liver failure, cirrhosis, and HCC. Therefore, antiviral therapy is crucial for the clinical management of CHB. Current antiviral drugs including nucleoside/nucleotide analogues (NAs) and interferon-α (IFN-α) can suppress HBV replication and reduce the progression of liver disease, thus improving the long-term outcomes of CHB patients. This chapter will discuss the standard and optimization antiviral therapies in treatment-naïve and treatment-experienced patients, as well as in the special populations. The up-to-date advances in the development of new anti-HBV agents will be also discussed. With the combination of the current antiviral drugs and the newly developed antiviral agents targeting the different steps of the viral life cycle or the newly developed agents modulating the host immune responses, the ultimate eradication of HBV will be achieved in the future.

1 Introduction

Chronic HBV infection remains the leading cause of liver-related morbidity and mortality across the world. CHB patients are at the risk of developing cirrhosis and HCC. The 5-year cumulative incidence of cirrhosis in untreated CHB patients is 8–20%, the 5-year cumulative risk of hepatic decompensation in cirrhotic patients is approximately 20%, and the annual rate of cirrhosis progressing to HCC is 2–5%

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[1, 2]. High serum HBV DNA level is associated with the increased risk of cirrhosis and HCC [3, 4]. Cirrhosis patients with low, but detectable, viral load are still at risk of HCC [5]. Thus, antiviral treatment with sustained suppression of HBV DNA is important to relieve underlying liver injury and prevent its progression toward cirrhosis and HCC.

2 Current Antiviral Therapy

2.1 Goals of Antiviral Treatment

The main goals of antiviral therapy are to improve the survival and the quality of life for CHB patients [6, 7]. Through sufficient antiviral treatment, sustained suppression of HBV replication can be achieved, which is associated with ALT normalization and liver histology improvement, thus leading to the reversal of fibrosis or even cirrhosis, and the decrease of hepatic decompensation and HCC [8, 9].

The ultimate goal of antiviral therapy is to cure HBV infection with the elimination of all forms of potentially replicating HBV, which is hardly achievable with current antiviral therapy due to the persistence of cccDNA (the HBV transcriptional template) in the hepatocyte nucleus [2]. However, a "functional cure" defined as the HBsAg loss or seroconversion and sustained HBV DNA suppression [10] is a realistic goal and can be achieved in a proportion of eligible CHB patients with optimization of antiviral strategies such as the different combinations of NAs and Peg-IFN.

2.2 Indications for Antiviral Treatment

The current indications to begin antiviral treatment are generally based on the serum HBV DNA, ALT levels, and the severity of liver disease (assessed by liver biopsy and/or noninvasive tests) (Table 1) [1]. Indications for antiviral treatment should also consider the patients' age, family history of cirrhosis or HCC, and concomitant diseases.

2.2.1 Antiviral Treatment Indications for Non-cirrhotic Patients

Current professional society guidelines recommend the initiation of antiviral treatment in non-cirrhotic CHB patients who are in the immune-active phase, which is defined by an elevation of ALT $\geq$ 2 ULN (upper limits of normal) or significant liver histological disease plus HBV DNA $\geq$ 20,000 IU/mL if HBeAg positive or HBV DNA levels $\geq$ 2000 IU/mL if HBeAg negative [1, 2, 11, 12] (Table 1). The 2018 AASLD guidelines recommended utilizing the ALT levels of 35 U/L for males and

Table 1 Indications for antiviral treatment in CHB patients among different guidelines

	HBeAg positive	HBeAg negative	Compensated cirrhosis	Decompensated cirrhosis
EASL 2017	HBV DNA >2000 IU/mL and ALT>ULN	HBV DNA >2000 IU/mL and ALT>ULN	Any detectable HBV DNA level	Any detectable HBV DNA level
	At least moderate liver necroinflammation or fibrosis	At least moderate liver necroinflammation or fibrosis	Regardless of ALT levels	Regardless of ALT levels
AASLD 2018	HBV DNA >20 000 IU/mL and ALT>2 × ULN	HBV DNA >2000 IU/mL and ALT>2 × ULN	Any detectable HBV DNA level Regardless of ALT levels	HBsAg-positive Regardless of HBV DNA level, HBeAg status, or ALT levels
	HBV DNA >20 000 IU/mL, ULN <ALT≤ 2 × ULN and significant histological disease by liver biopsy or noninvasive testing	HBV DNA >2000 IU/mL, ULN <ALT≤ 2 × ULN and significant histological disease by liver biopsy or noninvasive testing		
	Immune-active CHB with HBV DNA below thresholds, ALT≤2 × ULN and older age (>40 years) or family history of HCC or presence of extrahepatic manifestation	Immune-active CHB with HBV DNA below thresholds, ALT≤2 × ULN and older age (>40 years) or family history of HCC or presence of extrahepatic manifestation		
APASL 2015	HBV DNA>20 000 IU/mL and ALT>2 × ULN	HBV DNA>2000 IU/mL and ALT>2 × ULN	HBV DNA> 2000 IU/mL, regardless of ALT levels or detectable HBV DNA if elevated ALT levels	Any detectable HBV DNA level, regardless of ALT levels
	HBV DNA>20 000 IU/mL, ALT 1–2 × ULN, biopsy if noninvasive tests suggest evidence of significant fibrosis, ALT is persistently elevated, age>35 years or family h/o HCC or cirrhosis. Treat if moderate to severe inflammation or significant fibrosis	HBV DNA>20 000 IU/mL, elevated ALT≤ 2 × ULN, consider biopsy if age > 35 years or family history of HCC or cirrhosis. Treat of moderate to severe inflammation or significant fibrosis		
	HBV DNA< 20 000 IU/mL, consider biopsy if age > 35 years or family history of HCC or cirrhosis. Treat if moderate to severe inflammation or significant fibrosis	HBV DNA< 2000 IU/mL, consider biopsy if age > 35 years or family history of HCC or cirrhosis. Treat if moderate to severe inflammation or significant fibrosis		

(continued)

Table 1 (continued)

	HBeAg positive	HBeAg negative	Compensated cirrhosis	Decompensated cirrhosis
China 2015	HBV DNA>20 000 IU/mL and ALT>2 ×ULN	HBV DNA>2000 IU/mL and ALT>2 ×ULN	Any detectable HBV DNA, regardless of ALT and HBeAg status	Any detectable HBV DNA, regardless of ALT and HBeAg status
	HBV DNA>20 000 IU/mL and ULN <ALT≤ 2×ULN. Consider biopsy and noninvasive fibrosis tests; treat if significant inflammation or fibrosis exists	HBV DNA>2000 IU/mL and ULN <ALT≤ 2×ULN. Consider biopsy and noninvasive fibrosis tests; treat if significant inflammation or fibrosis exists		
	HBV DNA>20 000 IU/mL and normal ALT levels. Consider biopsy and noninvasive fibrosis tests if age > 30 years and family history of HCC or cirrhosis. Treat if moderate to significant inflammation or fibrosis exists	HBV DNA>2000 IU/mL and normal ALT levels. Consider biopsy and noninvasive fibrosis tests if age > 30 years and family history of HCC or cirrhosis. Treat if significant inflammation or fibrosis exists		

25 U/L for females as ULN rather than local laboratory ULN to guide the initiation of antiviral treatment [11]. It needs to be noted that CHB is a dynamic disease and individuals with CHB can transition through different phases with variable levels of HBV DNA, ALT, and HBV antigens, and thus a single ALT, HBV DNA level, and HBV antigens are insufficient to assign phase of infection and/or need for treatment. Serial testing of ALT, HBV DNA, and HBV antigen are required to guide the treatment decisions [11].

For patients unfulfilling the above treatment indications, especially in patients >30 years or with family history of cirrhosis or HCC, liver biopsy or noninvasive test (such as elastography) is recommended to assess the grade of hepatic inflammation or the evidence of fibrosis, thereby helping to determine whether it is necessary to start antiviral treatment. For instance, for patients with HBV DNA >2000 IU/mL and at least moderate fibrosis (assessed by liver biopsy or elastography), antiviral treatment may be initiated even if ALT levels are mildly elevated (ULN <ALT $\leq$ 2 ×ULN) or normal [13]. In addition, antiviral treatment is recommended for CHB patients with extrahepatic manifestations, such as dermatomyositis [14] and vasculitis [15], regardless of ALT level and HBeAg status.

Antiviral treatment is currently not recommended for CHB patients in the immune-tolerant phase, which is defined by persistently normal ALT, high levels of HBV DNA, biopsies showing the absence of significant inflammation or fibrosis, as well as younger age (typically below 30 years old) [16]. However, the likelihood of transitioning from immune-tolerant phase to HBeAg-positive immune-active phase increases with age; the EASL guidelines suggest that patients with normal ALT and high HBV DNA level but older than 30 years may be treated regardless of the severity of liver histological lesions [13]. The recommendation against antiviral treatment for immune-tolerant CHB patients is due to the following reasons: 1) the risk of disease progression in the immune-tolerant phase is very slow; and 2) the current antiviral treatment during this phase is associated with a minimal chance of suppressing HBV replication completely and the potential harms of antiviral drug side effects and development of drug resistance. However, if new and effective anti-HBV drugs for immune-tolerant CHB patients could be developed in the future, these patients may also be treated.

2.2.2 Antiviral Treatment Indications for Cirrhotic patients

For patients with compensated cirrhosis and detectable HBV DNA, indefinite antiviral therapy is recommended to reduce the risk of decompensation, regardless of ALT level and HBeAg status [1, 12, 17]. For HBsAg-positive patients with decompensated cirrhosis, the 2018 AASLD guidelines recommended indefinite antiviral therapy regardless of HBV DNA level, HBeAg status, or ALT level to decrease risk of worsening liver-related complications [11]. Indications for other special populations will be discussed in detail below.

2.3 Current Anti-HBV Drugs

Current anti-HBV drugs can be categorized into two classes: nucleoside/nucleotide analogues (NAs) and interferon-α (IFN-α) [2]. NAs are widely used due to its favorable safety profile, convenient route of administration, and no obvious contraindications as compared with IFN-α. However, the treatment duration for NAs is not definite, and long-term NA therapy may increase the risk of drug resistance, while for IFN-α, there is no drug resistance, and the treatment duration for CHB treatment is relatively definite. IFN-α treatment induces higher rate of HBsAg loss and HBeAg seroconversion compared to NAs. Nevertheless, the administration of IFN-α needs injection and is contraindicated in patients with decompensated cirrhosis or autoimmune disease, pregnant women, and patients with uncontrolled severe depression or psychosis. Thus, when designing an optimal therapy for individual patients, physicians should take account of many factors including patients' characteristics, the estimated duration of treatment, the side effects of chosen drugs, the treatment costs, and the drug resistance.

2.3.1 Nucleoside/Nucleotide Analogues

Antiviral therapy with NAs for CHB has become the primary standard treatment strategy. Currently, there are six NAs approved for CHB antiviral treatment: lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF). The development of NAs is ascribed to the comprehensive understanding of HBV replication process. NAs not only inhibit HBV reverse transcriptase activity but also compete with natural nucleotide substrates for incorporation into the elongating DNA chain, thus interrupting HBV DNA synthesis [18, 19]. Long-term NA therapy can decrease the cccDNA pool of infected hepatocytes by inhibiting the recycling of the nucleocapsids. However, NAs cannot prevent the initial cccDNA formation in newly infected hepatocytes [19].

LAM, ADV, and LdT are the first generation of NAs developed for anti-HBV treatment. These NAs have low barrier to resistance and therefore are liable to develop resistance during long-term treatment. LAM is the first nucleoside analogue approved by the US FDA in 1998 for the treatment of CHB. It competes for cytosine in the synthesis of viral DNA. CHB patients receiving 104 weeks of 100 mg LAM treatment showed 52% rate of virological response [20]. However, long-term LAM therapy led to high rate of drug resistance: 65–70% after 5 years of LAM therapy [21]. Following LAM, ADV was the second NA approved by the US FDA in 2002 for the treatment of CHB. It is a phosphonate acyclic nucleotide analogue of adenosine monophosphate. Following 1-year ADV therapy, the rates of virological response and HBeAg seroconversion were 21% and 12% in HBeAg-positive patients, respectively [22]. And in HBeAg-negative patients, the rates of virological

response and histological improvement were 51% and 64%, respectively [23]. However, long-term ADV treatment also leads to high drug resistance rate (20–29% after 5-year treatment) [24, 25]. LdT is another nucleoside analogue and is the unmodified β-L enantiomer of the naturally occurring nucleoside thymidine. The rates of virological response in HBeAg-positive and HBeAg-negative patients treated with 104 weeks of LdT were 55.6% and 82%, respectively [26]. With a proven safety profile, LdT is a pregnancy category B medication and has been applied to prevent mother-to-child-transmission (MTCT) in mothers with HBV infection [27–29]. However, similar with LAM and ADV, long-term LdT treatment leads to high rate of drug resistance (34% after 3-year LdT therapy) [30]. When drug-resistant mutations occur in CHB patients, the clinical benefit of treatment decreases, and hepatitis flares and even liver failure may occur. Therefore, selecting potent and low-resistant antiviral drugs is highly recommended for treatment-naïve CHB patients.

ETV, TDF, and TAF are potent antiviral NAs with high genetic barrier to HBV resistance, and they are recommended as the first-line oral anti-HBV drugs. ETV is a guanosine nucleoside analogue with selective activity against HBV and has been commercially available since 2005 [31]. The effective concentration (EC_{50}) of ETV is around 4 nM, which is at least 100-fold more potent than LAM or ADV on the suppression of HBV [32]. TDF is an acyclic nucleotide analogue with activity in vitro against retroviruses, including HIV and HBV. It is an orally bioavailable ester prodrug of tenofovir. TDF was approved by the US FDA for the treatment of CHB in 2008 and is categorized as a pregnancy category B drug. TAF is a newly approved drug for the antiviral treatment of CHB in 2017 [1]. It is a new prodrug of tenofovir and exerts more stable concentration in the serum than TDF. Compared with TDF, TAF permits a lower dose in circulating and less systemic exposure, thereby decreasing the renal and bone toxicity.

For the safety profiles of the NAs, TDF has proven to be associated with dose-dependent renal toxicity in animal studies [33]. The first case of TDF-associated nephrotoxicity was reported in 2002 in a patient with HIV [34]. Later, numerous case reports of TDF-induced nephrotoxicity have been published. In 2015, TDF-induced Fanconi syndrome was observed in a CHB patient [35]. This patient developed a progressive chronic kidney disease with serious hypophosphatemia and secondary osteomalacia. After TDF withdrawal and oral supplementation with phosphate and calcitriol, the renal damage gradually resolved. As for TAF, data from phase III registration trials demonstrated that it induced less reduction in the estimated glomerular filtration rate (eGFR) and bone mineral density than TDF [36]. Thus, all patients treated with potent NAs, especially TDF, should periodically monitor clinical indicators, including complete blood count, liver and kidney function tests, serum HBV DNA, and abdominal ultrasound. Liver function tests should be performed every 3–4 months during the first year and every 6 months thereafter. Serum HBV DNA should be determined every 3–4 months during the first year and every 6–12 months thereafter.

2.3.2 Peg-IFN-α

Interferons are central mediators of immune response to viral infections. IFN-α can induce IFN-stimulated genes (ISGs), exerting antiviral functions against a variety of viruses. IFN-α exhibits direct inhibition of HBV DNA replication and clears infected hepatocytes through indirect regulation of the host immunity [37]. As the covalent attachment of polyethylene glycol (Peg) molecules to conventional IFN-α produces a biologically active molecule with a longer half-life, pegylated interferon α (Peg-IFN-α) increasingly replaced conventional IFN-α with improved pharmacokinetic properties [38]. Thus, Peg-IFN-α has been selected as one of the first-line therapies to treat CHB patients. Of note, Peg-IFN-α is prohibited in patients with decompensated cirrhosis.

2.4 Treatment Strategies for Patients Chronically Infected with HBV

The choice of an optimal therapy for individual patient depends on several factors, including age, sex, stage of liver disease, coinfections, treatment duration, side effects, and drug resistance.

2.4.1 NAs for CHB Patients

Potent NAs with high barrier to resistance (ETV, TDF, and TAF) are recommended as the first-line antiviral drugs. Long-term ETV treatment showed good tolerance, a favorable safety profile. The rates of virological responses in HBeAg-positive and HBeAg-negative patients after 1-year ETV therapy were 67% and 90%, respectively [39, 40], and the HBeAg seroconversion was 21% in HBeAg-positive patients [39]. Five years of ETV therapy resulted in 99% cumulative rate of virological response and 53% rate of HBeAg loss in HBeAg-positive patients [41]. The virological response at year 5 reached 100% in HBeAg-negative patients [42]. Similarly, a study evaluating the efficacy of ETV in NA-naïve Egyptian patients reported that the rate of HBV DNA undetectability reached 100% after 5 years of ETV therapy [43].

Among treatment-naïve CHB patients with HBeAg positive and negative, the rates of virological response were 76% and 93% after receiving 48-week TDF, respectively [44], and the virological response increased to 97% in HBeAg-positive patients after 5 years. After 3 years, about 96% of HBeAg-negative patients treated with TDF achieved virological response [45]. Phase III studies comparing TAF to TDF in CHB patients demonstrated that with the anti-HBV efficacy of TAF was non-inferior to that of TDF, but TAF had a better safety profile than TDF in CHB patients [46, 47].

The NAs with low barrier to drug resistance, such as LAM, LdT, and ADV, should be avoided, as this may lead to inappropriate viral suppression and the emergence of multidrug-resistant strains. For treatment-experienced CHB patients with NAs of low barrier to resistance (LAM, ADV, LdT), it is recommended to change to a more potent drug without cross-resistance [1]. The risk of resistance is associated with high baseline HBV DNA levels, a slow decline in HBV DNA, and a previous suboptimal NA treatment [1]. Patients previously treated with LAM, LdT, or ADV often develop high rate of resistance during prolonged treatment. For ADV-experienced patients, high rates of CVR could be achieved after switching to ETV [48]. Although ETV is an excellent inhibitor of HBV reverse transcriptase, it often fails to treat LAM-resistant individuals. Patients carrying LAM-resistant virus strain showed a highly increased ETV-resistance rate (51% *vs.* 1.2% in treatment-naïve patients receiving 5-year ETV therapy) [49, 50], as LAM-resistant mutations contribute to the development of ETV resistance [51]. The LAM mutations, rtM204I/V with or without rtL180M, along with other mutations are frequently detected in patients with ETV resistance [32], and the presence of LAM-resistant mutations leads to several 100-fold increases in ETV resistance.

For NA-experienced patient, TDF also have antiviral efficacy to act as an idea agent for CHB patients with LAM or LdT resistance [52]. Both treatment-naïve and treatment-experienced patients showed a rapid decline in HBV DNA within 3 months of TDF initiation [53]. HBV DNA < 69 IU/mL was achieved in 91% of treatment-naïve patients and 96% of treatment-experienced patients, respectively, demonstrating that TDF showed a rapid and sustained suppression of HBV DNA in CHB patients, irrespective of treatment history. In ADV treatment-experienced CHB patients, TDF had inferior efficacy compared to NA-naïve patients. A total of 92.3% of NA-naïve patients and 84.5% of NA-exposed patients achieved CVR, respectively [54], suggesting that the response of patients with previous ADV switching to TDF monotherapy should be monitored closely.

For patients harboring multiple drug-resistant HBV strains, combination of TDF and ETV seems to be an effective and safe rescue approach [55, 56]. To reduce the emergence of multidrug-resistant strains, combination therapies, especially combination of NAs with low barrier to resistance, such as LAM or LdT with ADV, and sequential monotherapies with agents with a low barrier to resistance are not generally recommended, which may ultimately increase the difficulty and cost of treatment.

2.4.2 Peg-IFN-α for CHB Patients

Peg-IFN-α, an immunomodulatory agent, could enhance host immunity to mount a defense against HBV and modest antiviral action [12]. Peg-IFN-α therapy offers several benefits over NAs for treatment of CHB including a finite duration of therapy and higher rates of anti-HBe and anti-HBs seroconversion with 12 months of therapy [57].

Rate of HBsAg loss was 3–7% following 12 months of Peg-IFN-α treatment, higher than 12-month treatment with current NAs (1% for LAM, 0% for ADV, 2% for ETV, 0.5% for LdT, and 3% for TDF) [58]. With 12-month therapy of Peg-IFN-α for HBeAg-positive CHB patients, the rate of HBV DNA<60–80 IU/ml, anti-HBe seroconversion, ALT normalization, and HBsAg loss were 14%, 32%, 41%, and 3%, respectively [59]. The response rates of 48-week Peg-IFN-α treatment for HBeAg-negative CHB were also evaluated in another multicenter, randomized study; the rate of HBV DNA <60–80 IU/ml, ALT normalization, and HBsAg loss were 19%, 59%, and 4%, respectively [60]. HBsAg loss rarely occurred during Peg-IFN-α therapy in HBeAg-negative CHB patients, but the rate of HBsAg loss progressively increased from 3% at month 6 to 9% at year 3 to 12% at year 5 after Peg-IFN-α discontinuation [58]. IFN-α treatment improved long-term outcomes, including decreased risk of hepatic complication survival and HCC and in CHB patients with sustained response [12, 61]. A 5-year observation cohort study revealed Peg-IFN-α-treated patients showed a lower cumulative incidence of cirrhosis and HCC [62].

The evaluation of predictors for response before and during treatment is very important for CHB patient with Peg-IFN-α therapy. For patients with HBeAg positive, having low HBV DNA (below 2×10^8 IU/mL), genotype A, as well as high serum ALT levels (above 2–5 times ULN) and high activity scores on liver biopsy, Peg-IFN-α could be considered as first-line antiviral agent [1, 58]. For patients with HBeAg negative, genotype D, a combination of no decrease in HBsAg levels and 2 $\log_{10}$ IU/ml reduction of HBV DNA at 12 weeks of Peg-IFN-α therapy predicts no response and should be used as Peg-IFN-α stopping rules [1, 58]. These treatment predictors for the existing antiviral therapies at various time points may be useful to guide initiation and continuation of Peg-IFN-α therapy.

Screening suitable patients prior to treatment is quite important for Peg-IFN-α therapy. Relative or absolute contraindications to IFN-α treatment include Child B/C cirrhosis, cirrhotic hypersplenism, autoimmune hepatitis, hyperthyroidism, coronary artery disease, renal transplant, pregnancy, seizures, severe depression, etc. The side effects of IFN-α are relatively common but are acceptable in most patients. The adverse effects of IFN-α mainly include flu-like symptoms, fatigue, bone marrow suppression, and exacerbation of autoimmune illnesses [19]. Therefore, patients should be closely monitored throughout the therapy. Complete blood counts and serum ALT levels should be monitored monthly, and TSH should be monitored every 3 months. Serum HBV DNA and HBsAg in all CHB patients and HBeAg and anti-HBe in HBeAg-positive patients should be examined at 3, 6, and 12 months of Peg-IFN-α treatment and at 6 and 12 months posttreatment. Patients with the definite therapy course of Peg-IFN-α, despite HBV DNA negative and serological conversion at the end of treatment, require long-term follow-up in case of HBV reactivation. In summary, IFN-α is associated with a broad spectrum of potential adverse effects, and the recommendations to use Peg-IFN-α should balance benefits versus risks, and decisions should be made according to individual patient characteristics and preference.

2.4.3 Combination of NA Plus Peg-IFN-α Therapy for CHB Patients

The current anti-HBV therapy with potent and high genetic barrier NAs can suppress the viral replication to undetectable level in the blood circulation in the majority of CHB patients, preventing the progression of CHB to cirrhosis and markedly decreasing the rates of HBV-related HCC. However, current long-term anti-HBV NAs can rarely achieve the "functional cure" of HBV (HBsAg loss or seroconversion), the best current stopping rule. This goal is hardly achievable by the finite-duration treatment with Peg-IFN, either. Hence, to accomplish the goal of "functional cure" of HBV infection in more CHB patients, the combination of a potent NA with Peg-IFN-α has been investigated. The rationale behind is that the two classes of anti-HBV agents have different mechanism of actions, the advantages of the potent antiviral effect of the NAs and the immunomodulating effect of the Peg-IFN-α, and thus their combination would conceptually result in a synergistic anti-HBV effect.

There are two different ways to combine NA and Peg-IFN: 1) the de novo combination, which means the simultaneous administration of the two agents in treatment-naïve CHB patients, and 2) the sequential combination, which means the "add-on" or "switch-to" strategy to CHB patients who are already on treatment with either drug (Table 2).

de novo Combination Therapy

The initial treatments of LAM plus Peg-IFN and ADV plus Peg-IFN showed less-than-desirable results in treatment-naïve patients [59, 60, 63]. The combination therapy with LdT plus Peg-IFN is prohibited due to a high risk of severe polyneuropathy [58, 64]. The de novo combination of TDF and administration of Peg-IFN have been recently investigated in a global multicenter randomized controlled study (Marcellin) [65]. In this study, 740 treatment-naïve patients with HBe-positive and HBe-negative CHB were randomly assigned to receive TDF plus Peg-IFN-α2a for 48 weeks (group A), TDF plus Peg-IFN-α2a for 16 weeks followed by TDF for 32 weeks (group B), TDF for 120 weeks (group C), or Peg-IFN-α2a for 48 weeks (group D). The rates of HBsAg loss at week 72 (24 weeks posttreatment) in the four groups were 9.1%, 2.8%, 0%, and 2.8%, respectively. In the follow-up study at week 120 (72 weeks posttreatment), the rates of HBsAg loss in the combination group increased from 9.1% to 10.4% [66]. Thus, patients receiving combination of TDF plus Peg-IFN had a higher rate of HBsAg loss than those receiving Peg-IFN or TDF alone. Although the increased rate of HBsAg loss in patients receiving TDF plus Peg-IFN therapy was encouraging, the overall rate of HBsAg at week 120 (10.4%) in the combination group was still relatively low, meaning that approximately 90% of patients did not achieve a sustained immune control. Besides, the benefit of the increased HBsAg loss was mainly associated with HBV genotype A and treatment with TDF plus Peg-IFN in the study [67].

Table 2 Combination of NA plus Peg-IFN therapy for CHB patients

De novo combination therapy

Study design	Country	Year of publication	No. of patients	Type of CHB (HBeAg positive/ negative)	Follow-up	NAs	HBeAg seroconversion (%)	The HBsAg loss (%)	Reference
A secondary analysis of data from study GSUS-174-0149, randomized, open-label, active-controlled, superiority trial (NCT01277601)	Global study of 30 investigators	2016	740	428/312	72 weeks	TDF	NA	6.5%	[65]
Randomized, open-label, active-controlled, multinational, superiority trial (NCT01277601)	Global study of 149 investigators	2016	740	428/312	72 weeks	TDF	25%	9.1%	[67]
Open-label, active-controlled study (NCT01277601)	Global study of 19 investigators	2018	740	428/312	120 weeks	TDF	29.5%	10.4%	[66]
Retrospective study (ISRCTN 77073364)	The Netherlands	2017	92	44/48	5 years	ADV	22%	18%	[63]
Multicenter, randomized, partially double-blind study	67 sites in 16 countries	2005	814	814/0	72 weeks	LAM	32%	3%	[59]
Multicenter, randomized, partially double-blind study	54 sites in 13 countries	2005	552	0/552	72 weeks	LAM	NA	2.8%	[60]
Randomized controlled, open-label trial (NCT00973219)	The Netherlands	2017	151	0/151	72 weeks	TDF or ADV	NA	4%	[68]

Sequential combination therapy									
Study design	Country	Year of publication	No. of patients	Type of CHB (HBeAg positive/ negative)	Follow-up	NAs before switch-to IFN or combination	HBeAg seroconversion (%)	The HBsAg loss (%)	Reference
Open-label, multicenter, randomized controlled trial (NCT00877760)	14 centers in 5 countries in Europe and Asia	2015	185	185/0	96 weeks	ETV	26%	1.18%	[72]
Randomized, controlled, open-label trial (NCT01172392)	France	2017	185	0/185	96 weeks	ETV or TDF or ADV or LAM	NA	7.8%	[69]
Open-label, randomized study (NCT00940485)	China	2014	200	200/0	48 weeks	ETV	14.9%	8.5%	[70]
Open-label, randomized study (NCT01464281)	China	2018	303	0/303	96 weeks	ADV/ETV/ LAM	NA	48 weeks 14.4% 96 weeks 20.7%	[71]

However, a recent randomized controlled, open-label study did not support the use of combination treatment with Peg-IFN and NA in patients with CHB [68]. At week 72, only two patients (4%) in the Peg-IFN plus TDF group and two patients in the Peg-IFN plus ADV group achieved HBsAg loss, compared with none of the patients in the no-treatment group (p=0.377). All four patients with HBsAg loss were included in the group of patients with HBV DNA less than 2000 IU/mL, so the baseline HBV DNA should be taken into account before initial de novo combination therapy.

Sequential Combination Therapy

Sequential combination therapy (including "add-on" and "switch-to" strategy) may be alternative options for CHB patients pursuing a functional cure. Starting with an NA first and then followed by Peg-IFN add-on seems to be a very logic approach to the sequential combination strategy. The concept is that the administration of a potent NA first would quickly halt the viral replication and therefore partially restore the host adaptive immune response, whereas the Peg-IFN add-on later may enhance serological response rates, resulting in more patients achieving a functional cure of CHB [69–71].

The study by Brouwer et al. (ARES study) investigated the "early add-on" strategy by comparing 24 weeks of ETV followed by 24 weeks of Peg-IFN add-on versus 48 weeks of ETV monotherapy for treatment-naïve HBeAg-positive CHB patients [72]. It showed that Peg-IFN add-on therapy led to a higher proportion of HBeAg serological response compared to ETV monotherapy. At week 48, the response defined as HBeAg loss with HBV DNA <200 IU/mL was achieved in 16 of 85 (19%) patients receiving the combination therapy versus 9 of 90 (10%) patients receiving ETV monotherapy. At week 72 (24 weeks posttreatment), the response rate in the combination group increased to 32% (27/85). However, in the ARES long-term follow-up study (the median follow-up duration was 226 weeks), the rates of serological response became comparable between the combination group and the ETV monotherapy group, suggesting that Peg-IFN add-on may lead to accelerated HBeAg loss rather than increased long-term HBeAg loss [73].

The "late add-on" Peg-IFN combination therapy was recently investigated in a multicenter and randomized trial enrolling only HBeAg-negative CHB patients with undetected HBV DNA by at least 1 year of NA treatment (PEGAN study) [69]. In this study, 183 patients were randomized to either continue NA or add on Peg-IFN treatment for 48 weeks. Due to the adverse effects of Peg-IFN, only 65 out of 90 patients in the Peg-IFN-α add-on group completed a full 48-week course of Peg-IFN-α. As the primary endpoint for this study was HBsAg loss at week 96 by intention-to-treat analysis (8% in the Peg-IFN add-on group versus 3% in the NA group, $P = 0.15$), the interpretation of the study results was that Peg-IFN-α add-on was poorly tolerated [69]. However, HBsAg loss rates were significantly higher in the full-dose Peg-IFN-α add-on group than in the NA group, being 11% *vs.* 0%, 11% *vs.* 3%, and 14% *vs.* 4% at week 48, week 96, and week 144, respectively [69].

Secondary post hoc analysis showed that patients who had lower baseline HBsAg titers might benefit more from this add-on strategy to achieve HBsAg loss and anti-HBs seroconversion [69].

The rates of HBsAg loss in the "switch-to" Peg-IFN-α strategy have also been investigated in CHB patients pre-treated with NA. In the "early switch-to" study (OSST trial), 192 HBeAg-positive patients receiving 9 to 36 months of ETV therapy with HBeAg <100 PEIU/ml and HBV DNA≤1000 copies/ml were randomized 1:1 to receive ETV or switch to Peg-IFN-α2a for 48 weeks. At week 48, serological response rates were significantly higher in the Peg-IFN-α group than the ETV group (HBeAg seroconversion 14.9% *vs.* 6.1%; HBsAg loss 8.5% *vs.* 0%) [70]. The study further found that a baseline HBsAg level <1500 IU/ml as the optimal cutoff to predict HBsAg loss and week 12 HBsAg <200 IU/ml were associated with the highest rates of HBsAg loss 77.8% (7/9).

In the "late switch-to" study (New Switch trial), 305 HBeAg-positive patients who achieved HBeAg loss and HBV DNA <200 IU/mL with previous NA treatment (ADV, LAM, or ETV) were randomized 1:1 to receive Peg-IFN for 48 or 96 weeks [71]. The rates of HBsAg loss were achieved in 14.4% (22/153) of patients receiving "switch-to" Peg-IFN for 48 weeks and in 20.7% (31/150) of patients receiving "switch-to" Peg-IFN for 96 weeks. Similar to the OSST study, the New Switch study also found that baseline HBsAg <1500 IU/mL and week 24 HBsAg <200 IU/mL were associated with the highest rates of HBsAg loss 51.4% (18/35) and 58.7% (27/46) at the end of both 48- and 96-week treatment, respectively [71].

In summary, recent studies have demonstrated that the combination of NA with Peg-IFN either simultaneously or sequentially can enhance the rates of HBsAg loss, but the benefits are mainly limited to a relatively small proportion of patients, especially in those with low baseline HBsAg level and on-treatment HBsAg response. Therefore, CHB patients who can benefit from NA and Peg-IFN combination therapy should be carefully evaluated including age, viral load, genotype, baseline ALT, and HBsAg levels. In addition, Peg-IFN stopping rules based on on-treatment HBsAg kinetics should be followed during the treatment to make decisions whether to continue or discontinue Peg-IFN and shift to NA monotherapy. Further investigations are needed to identify the optimal Peg-IFN combination strategy and the subgroup of CHB patients with the highest potential to benefit from the combination treatment.

2.4.4 Treatment Strategies for Special Populations

Patients with HBV-Related Cirrhosis

Patients with evidence of HBV-related cirrhosis and detectable HBV DNA are strong indicators for antiviral treatment, regardless of ALT levels and HBeAg status. The aim of antiviral therapy for patients with compensated cirrhosis is to reduce the risk of disease progression to hepatic decompensation and HCC. It has been demonstrated that the occurrences of death, hepatic decompensation, and HCC were

less frequent in the treated cohort than in the untreated controls, with the 5-year cumulative incidences being 19.4% *vs.* 43.9%, 15.4% *vs.* 45.4%, and 13.8% *vs.* 23.4%, respectively [74]. Taking both efficacy and drug resistance profiles into account, antiviral drugs for HBV-related cirrhosis should be safe and affordable for long-term use to achieve a high rate of sustained HBV suppression with a low risk of drug resistance. Therefore, potent and low drug-resistant NAs (ETV, TDF, and TAF) are preferred for these patients. It has been reported that more than 2 years of ETV treatment for cirrhotic patients led to the improvement of liver function and fibrosis markers [75]. Moreover, up to 5 years of treatment with TDF achieved high rates of hepatic fibrosis regression and even the reversion of cirrhosis [76]. It needs to be noted that although Peg-IFN-α is not contraindicated in patients with compensated cirrhosis, it should be used with caution due to its side effects, and therefore the safer NAs are preferred and recommended [2].

Antiviral therapy in patients with decompensated cirrhosis has been shown to slow disease progression and may delay the burden of liver transplantation. Patients with decompensated cirrhosis and detectable HBV-DNA should be treated urgently, while HBsAg-positive decompensated patients with undetectable HBV-DNA may also receive lifelong antiviral therapy to reduce the risk of aggravation of liver-related complications [11]. ETV and TDF are recommended as the first-line NAs in these patients. Treatment with either ETV or TDF in patients with decompensated cirrhosis improved both hepatic function and Child-Turcotte-Pugh (CTP) and Model for End-Stage Liver Disease (MELD) scores [77, 78]. Although data concerning the use of TAF in these patients are currently insufficient, TAF may also be used in patients with decompensated cirrhosis due to its favorable safety profile. It needs to be noted that long-term antiviral therapy decreases but does not eliminate the risk of HCC in patients with liver cirrhosis [79–81], thus long-term surveillance of HCC still required even with successful antiviral treatment in these patients.

Patients with HBV-Related HCC

High serum HBV DNA is associated with early HCC recurrence after curative resection in patients with HBV-related HCC [82]. In addition, reactivation of HBV may be induced by HCC treatment strategies including curative resection, radiofrequency ablation (RFA), trans-arterial chemoembolization (TACE), and radioembolization [83]. Therefore, antiviral therapy is an important part of the comprehensive treatment for HBV-related HCC. NA treatment following HCC curative resection reduces HCC recurrence and improves the overall survival of patients with advanced HBV-related HCC [84]. Both ETV and TDF are recommended as the first-line antiviral agents for HBV-related HCC patients [17]. As TAF is the latest NA approved for anti-HBV treatment, the experience in its use for HBV-related HCC patients is currently limited. However, with its potent antiviral efficacy and favorable safety profile, it may also be considered for these HBV-related HCC patients.

Patients with Liver Failure Related with HBV Infection

Liver failure is a life-threatening disease with high short-term mortality [85]. It may develop following acute HBV infection or reactivation of chronic HBV infection. HBsAg-positive or HBV DNA-positive patients with liver failure (including acute, subacute, or acute on chronic) should consider NA therapy as soon as possible [86]. ETV, TDF, or TAF are the preferred NAs to improve the survival of CHB patients with liver failure [87, 88]. The beneficial effects were mostly observed in patients with MELD score within 20–30, while the mortality rate in patients with MELD score over 30 is >90% even with prompt antiviral treatment, and thus urgent liver transplantation should be considered in these patients [89].

Patients Undergoing Liver Transplantation Related with HBV Infection

In western countries, only 5–10% of liver transplantation is conducted for patients with HBV-related liver diseases, while in China, approximately 90% of the liver transplantation is due to HBV-related liver diseases [90]. HBV recurrence was a major problem for liver transplantation in the past, and patients with a high HBV viral load preoperatively had a higher risk of HBV reinfection after liver transplantation [91]. Previously, prophylactic therapy with HBIG only showed unfavorable results [92, 93]. With the advent of NA and its combination with HBIG, the risk of the HBV reinfection rate has been reduced to less than 5% [1]. However, the use of HBIG is costly and inconvenient (requires regular parenteral/intramuscular injections). In the current era of potent NAs with high barrier to drug resistance (ETV, TDF, and TAF), the prophylactic therapy with short course and low dose of HBIG or even HBIG-free regimen has been evaluated [93].

In 42 CHB patients with HBV DNA levels <100 IU/mL at the time of liver transplantation, prophylaxis using HBIG (5000 IU daily) intravenously in the anhepatic phase of liver transplantation and then daily for 5 days postoperatively (6 doses total) in combination with long-term NA therapy was highly effective in preventing HBV recurrence, with only 1 patient having detectable HBV DNA at 5 years after liver transplantation [94]. In another Greek study, 28 HBV-related cirrhotic patients with undetectable HBV DNA at the time of liver transplantation, prophylaxis using HBIG (1000 IU IM/day) for 7 days and then monthly for 6 months (13 doses total) plus ETV (n = 11) or TDF (n = 7) was also highly effective with all patients remaining HBsAg/HBV DNA negative during the follow-up period (9–43 months) [95]. A recent study from the University of Hong Kong has shown that HBIG-free prophylaxis using ETV monotherapy for CHB patients after liver transplantation is highly effective at preventing HBV reactivation [96]. In 265 consecutive CHB liver transplant recipients treated with ETV monoprophylaxis without HBIG, 85%, 88%, 87.0%, and 92% remained HBsAg negative at 1, 3, 5, and 8 years of follow-up, respectively, and 100% had undetectable HBV DNA at 8 years after transplantation. Of note, more than 60% of the 265 CHB liver transplant recipients had detectable HBV DNA at the time of liver transplantation. The overall 9-year survival was 85%

without any graft loss or death due to HBV reactivation [96]. Thus, short-course and low-dose HBIG in combination with potent NA or even HBIG-free NA monotherapy can be effective prophylaxis in the prevention of HBV reinfection after liver transplantation.

Of note, both the 2017 EASL guidelines and the 2018 AASLD guidelines stated that CHB patients with HDV and HIV coinfections were at high risk of HBV recurrence, and therefore the lifelong combination of HBIG and NA therapy was recommended as prophylaxis for these patients undergoing liver transplantation [1, 11]. Because of high potency and low rate of resistance with long-term use, ETV, TDF, and TAF are the preferred antiviral drugs for the prophylactic therapy, which should be administered in all CHB patients on the transplant waiting list. After liver transplantation, the duration of the antiviral therapy should be indefinite. After liver transplantation, the duration of the antiviral therapy should be indefinite, regardless of HBsAg, HBeAg, or HBV DNA status [1, 2, 97].

Patients Undergoing Immunosuppressive Therapy or Chemotherapy

After HBV exposure, the virus persists in the liver and other extrahepatic sites for long periods and may reactivate in individuals who receive immunosuppression or chemotherapy [98]. HBV reactivation is characterized by increased serum HBV DNA compared with the baseline level in HBsAg-positive patients or reverse seroconversion from HBsAg negative to HBsAg positive in HBsAg-negative and anti-HBc-positive patients. HBV reactivation causes elevation of ALT and hepatitis flare, which may result in liver failure and even death [99]. Thus, all patients should be screened with HBV markers including HBsAg, anti-HBs, and anti-HBc, prior to immunosuppressive therapy or chemotherapy, particularly in countries or regions with intermediate or high prevalence of HBV. HBsAg-positive patients are at high risk of HBV reactivation and should receive antiviral prophylaxis before immunosuppressive therapy or chemotherapy regardless of the baseline HBV DNA. Prophylactic antiviral therapy should be better initiated 1 week before or at the latest, concurrently at the initiation of immunosuppressive therapy [100]. Potent NAs (ETV, TDF, and TAF) should be preferred for the antiviral prophylaxis for CHB patients undergoing immunosuppressive therapy or chemotherapy [11].

Patients with HBsAg negative and anti-HBc positive are still at risk of HBV reactivation when they receive high-risk treatments, such as immunosuppressive agent rituximab and bone marrow/stem cell transplantation. Prophylactic anti-HBV drugs are recommended for these patients [12, 101]. HBsAg-negative and anti-HBc-positive patients who receive moderate- or low-risk immunosuppressive agents need to be regularly monitored with HBsAg and/or HBV DNA every 1–3 months during and after immunosuppression. Anti-HBV prophylaxis can be initiated at the first sign of HBV reactivation.

Regarding the duration of antiviral prophylaxis, the 2018 AASLD guidelines suggested that antiviral therapy should be at least 6 months (or at least 12 months for patients receiving rituximab) after completion of immunosuppressive therapy [2], whereas EASL recommends the antiviral therapy to be at least 12 months

(18 months for patients receiving rituximab) after cessation of the immunosuppressive treatment [1]. It is suggested that liver function and HBV DNA level should be routinely monitored every 3 to 6 months during prophylaxis and for at least 12 months after NA withdrawal.

Children with HBV Infection

Annually, about 2 million new HBV infections occur in children younger than 5 years old [102]. Exposed infants should be tested for HBsAg at 6–12 months after birth [103]. Most children with chronic HBV infection are in the immune-tolerant phase characterized by high viral load and normal ALT levels and respond poorly to currently available antiviral therapies. Thus, the 2018 AASLD guidelines recommend against the use of antiviral therapy in HBeAg-positive children with persistently normal ALT, regardless of HBV DNA level [2]. Although a rather benign course of CHB during childhood, about 3–5% and 0.01–0.03% of chronic carriers still have a risk of developing cirrhosis or HCC before adulthood, respectively [104]. Therefore, lifelong follow-up is recommended even for inactive carriers, because of the risk of cirrhosis and HCC and reactivation of HBV infection [104]. For children with normal ALT, monitoring should be done every 6 months, and the surveillance of HCC with liver ultrasound is recommended to be performed every 6–12 months, depending on the stage of fibrosis.

In children, the course of the HBV-related liver disease is generally mild, and most of the children do not meet the standard treatment indication. Thus the initiation of the antiviral treatment should be considered with caution [1]. CHB children fulfilling the indication for antiviral treatment should be treated [1, 11]. The antiviral therapy should be immediately initiated for CHB children with advanced liver diseases and cirrhosis [12].

There are several antiviral drugs approved for children with CHB, including conventional IFN-α (≥1 year old), LAM (≥2 years old), ETV (≥2 years old), and TDF (≥12 years old) [11]. The dose and treatment duration for each drug are shown in Table 3. Conventional IFN-α treatment accelerates ALT normalization, HBeAg

Table 3 Antiviral drugs approved for children with chronic HBV infection

Drugs	Ages approved for drug use	Dose	Duration
IFN-α	≥ 1 year	6 MU/m^2 three times per week	6 months
LAM	≥ 2 years	3 mg/kg/day	≥ 1 year
ADV	≥ 12 years	10 mg daily	≥ 1 year
ETV	≥ 2 years	10–30 kg, 0.015 mg/kg/day (maximum 0.5 mg); >30 kg, 0.5 mg daily	≥ 1 year
TDF	≥ 2 years[a]	300 mg daily	≥ 1 year
TAF	≥ 12 years	25 mg daily	≥ 1 year

[a]The European Medicines Agency approves TDF for children ≥2 years, and the US FDA approves for children ≥ 12 year

seroconversion, and viral load reduction in children [105, 106]. Although LAM is permitted for the treatment of CHB children, long-term use of LAM induces drug resistance and subsequent viral breakthrough [107], while ETV or TDF monotherapy has the advantage of high potency and low drug resistance [108, 109]. Thus, ETV and TDF should be preferred [11]. According to the 2017 EASL guidelines, TAF can be used in children ≥12 years old [1]. However, the 2018 AASLD guidelines stated that "TAF has not been studied in children. Thus, there are insufficient data to recommend the use of TAF in children 12 years and older" [11]. For children receiving antiviral treatment, the frequency of monitoring for safety, adherence, and efficacy of drugs should be determined on an individual basis.

Pregnancy with Chronic HBV Infection

When formulating treatment plan for women with CHB at childbearing age, the physician should take account of the effects and safety profile of different antiviral drugs [110]. Among current oral antiviral drugs, LdT and TDF are pregnancy category B medicines and are recommended for use in pregnant women with CHB, while ADV and ETV are pregnancy category C drugs and therefore are limited for use during pregnancy [1]. Although LAM is classified as pregnancy category C medicine, it can also be used in pregnant women with the safety data obtained from its use in pregnant women with HIV. Previous studies proved either LAM [111], LdT [27, 112], or TDF [113] effectively reduced perinatal HBV transmission. However, TDF is preferred with a better resistance profile and more safety data in pregnant women with chronic HBV infection. The 2018 AASLD guidelines stated that "TAF has not been studied in pregnant women. Thus, there are insufficient data to recommend the use of TAF in pregnancy" [11]. Peg-IFN-α is contraindicated for use during pregnancy.

The criteria to initiate antiviral therapy for women at childbearing age are the same as any other individuals with CHB. For women who fulfill the treatment indication and plan a pregnancy in the near future, NAs of category B (especially TDF) are recommended. For CHB patients on NA therapy who become pregnant, category B NAs can be continued (TDF is preferred), while category C NAs should be switched to TDF [2, 17].

Without intervention, 80–90% of infants exposed to HBV during the perinatal period may develop CHB infection [114]. Passive-active immunoprophylaxis, including HBIG and HBV vaccination at birth followed by two additional HBV vaccines within 6 months, is very effective against neonatal HBV exposure, reducing the MTCT rate from 90% to 10% [58]. It should be noted that about 10% immunoprophylaxis failures occur, which are almost exclusively in HBeAg-positive women with high HBV DNA levels.

Although CHB patients in immune-tolerant phase are generally not the indications for current antiviral therapy, accumulating evidences have suggested pregnant women with high HBV DNA level in immune-tolerant phase need antiviral therapy to reduce the risk of MTCT [28, 115–117]. The 2017 EASL and the 2018 AASLD

Table 4 Antiviral therapy to prevent mother-to-child transmission during pregnancy among different guidelines

	EASL 2017	AASLD 2018	China 2015	APASL 2015 update
Screening	Screening for HBsAg in the first trimester of pregnancy is strongly recommended	All pregnant women should be screened for HBsAg, especially those with high risk of HBV infection	-	Antenatal screening HBV in pregnant females is an evidence-based standard of practice
Standard indication	HBV DNA> 200 000 IU/mL or HBsAg levels >4 $\log_{10}$ IU/mL	HBV DNA> 200 000 IU/mL	HBV DNA> 2000 000 IU/mL	HBVDNA > 6–7 $\log_{10}$ IU/mL
Licensed antiviral drugs	TDF and LdT	LAM, LdT, and TDF	LAM, LdT, and TDF	TDF and LdT
	TDF is a preferred choice	TDF is a preferred choice		
The time to start therapy	Starting at weeks 24–28 of gestation	From 28 to 32 weeks of gestation	Starting at weeks 24–28 of gestation	From 28 to 32 weeks of gestation
The time to stop therapy	Continue therapy for up to 12 weeks after delivery	Antiviral therapy is discontinued at birth to 3 months postpartum	Antiviral therapy is discontinued at birth to months postpartum	The NAs could be stopped at birth and when breast-feeding starts. For those with ALT flares detected during the treatment period, continuation of antiviral treatment according to maternal liver disease status may be indicated
Breast-feeding after delivery	Breast-feeding is not contraindicated during maternal NA treatment	Breast-feeding is not prohibited during maternal NA treatment	Breast-feeding is recommended after drug withdrawal	Breast-feeding is discouraged during maternal NA treatment

guidelines recommended that pregnant women with serum HBV DNA > 20,0000 IU/ML should receive antiviral therapy to prevent MTCT [1, 2]. The time to start antiviral treatment to decrease the risk of MTCT varies among different guidelines [1, 11, 17]. Most guidelines recommended to initiate anti-HBV therapy at 24–28 weeks of gestation [1, 17], whereas the 2018 AASLD guidelines recommended at 28–32 weeks [11] (Table 4). Antiviral therapy may stop at delivery or continue for 12 weeks after delivery but should be closely monitored for ALT flares every 3 months for 6 months. As the concentration of TDF in breast milk is minimal with very limited bioavailability, breast-feeding is not contraindicated in HBsAg-positive mothers treated with TDF [1, 2, 12, 17].

Patients Coinfected with HBV and HCV

In CHB patients coinfected with HCV, the risks of the development of cirrhosis and HCC are higher than those with either HBV or HCV mono-infection [118, 119]. The treatment of HBV/HCV coinfection should be individualized based on the HBV and HCV viral loads, ALT levels, and liver fibrosis or cirrhosis assessment.

Anti-HCV therapy is indicated in coinfected patients with positive HCV-RNA. In the IFN-α era, the application of IFN-α plus ribavirin could achieve HCV eradication and HBV suppression in coinfected patients. With the advent of direct-acting antiviral (DAA), IFN-α-free and ribavirin-free DAA treatment has become the mainstream therapy for HCV infection. However, there is a potential risk of HBV reactivation during DAA therapy of patients with HCV/HBV coinfection, and life-threatening consequences have been reported in some individuals [120]. Therefore, during anti-HCV therapy with DAA, coinfected patients should be closely monitored by checking the HBV viral load and ALT levels [121].

In those HBV-/HCV-coinfected patients meeting the standard criteria for HBV treatment, HBV antiviral therapy should be started concurrently with DAA therapy of HCV [1]. The 2018 AASLD guidelines recommended monitoring HBV DNA levels every 4–8 weeks during DAA therapy and for 3 months post DAA treatment in HBsAg-positive patients who do not meet treatment criteria of anti-HBV treatment [11]. The risk of HBV reactivation in patients with HBsAg negative and anti-HBc positive is very low during HCV DAA therapy, but HBsAg and HBV DNA should be tested if ALT levels increase during or after anti-HCV therapy [11, 122]. For those HBV-/HCV-coinfected patients with cirrhosis, HBV antiviral therapy with NAs should be initiated concurrently with DAA therapy [121].

Another important issue that requires special attention is drug-drug interactions (DDI) in the context of combination therapy with NAs and DAAs. Based on the www.hep-druginteractions.org website, ETV can be safely co-administered with currently approved DAAs as no potential clinically significant DDI are indicated, but careful monitoring is still necessary during the therapy. Co-administration of TDF with DAA is either contraindicated or needs dose adjustment and additional monitoring [122].

Patients Coinfected with HBV and HIV

HBV/HIV coinfection induced increased risk of all-cause mortality and liver-related mortality [123]. HIV infection leads to higher HBV DNA levels, lower rates of HBeAg loss, and faster progression to cirrhosis [124].

The current guidelines recommend immediate initiation of antiretroviral therapy (ART) for all people living with HIV, regardless of CD4 cell count [125]. LAM, emtricitabine (FTC), TDF, and TAF are NAs with effective activities against both HIV and HBV. Thus, for patients with HBV/HIV coinfection, the ART regimen should include TDF or TAF plus LAM or FTC as the ART backbone [11]. Of note, these drugs should not be used as a single agent for HBV treatment in HBV-/HIV-

coinfected patients because of the risk of HIV resistance. Patients who are already on effective ART regimen that does not include drugs with antiviral activity against HBV should have the ART regimen altered to include TDF or TAF plus LAM or FTC. It needs to be noted that HBV-/HIV-coinfected patients with liver cirrhosis and low CD4 cell count require careful surveillance of immune reconstitution syndrome and subsequent liver decompensation in the first months after starting ART [1].

2.5 Endpoint of Antiviral Treatment

Deciding the antiviral treatment duration or therapy endpoint for CHB patients is challenging and needs to take account of many factors including the choice of antiviral agents (IFN-α-based or NA-based), sustained suppression of HBV DNA replication, HBeAg status, HBsAg status, and the presence of cirrhosis (compensated or decompensated).

For non-cirrhotic CHB patients, the duration of Peg-IFN-α treatment is generally 48 weeks, whereas the duration of NA-based antiviral therapy is variable and difficult to decide. For non-cirrhotic HBeAg-positive patients receiving NA therapy who have achieved persistent virological response, biochemical response, and serological response (HBeAg loss or seroconversion), discontinuation of NA therapy may be considered if the therapeutic responses persist during the consolidation treatment. However, the time of consolidation treatment varies, for at least 12 months in the 2018 AASLD [2] guidelines and for at least 3 years in Chinese CHB treatment guidelines [17]. However, due to the potential risk of virus relapse after NA withdrawal, close post-NA monitoring is still needed. For non-cirrhotic HBeAg-negative patients, the duration of anti-HBV treatment is unclear as the rate of HBV relapse is high in these patients, and therefore the long-term antiviral treatment is required. For both HBeAg-positive and HBeAg-negative patients with cirrhosis, NAs should be treated indefinitely.

HBsAg loss, with or without seroconversion to anti-HBs, termed as "functional cure," is considered as the optimal treatment endpoint for both HBeAg-positive and HBeAg-negative CHB patients. As serum HBsAg levels parallel the expression of cccDNA (the viral persistence reservoir), HBsAg loss represents a complete suppression HBV, low risk of HBV recurrence, and an improved long-term outcome. However, cirrhosis and HCC may still occur in patients with HBsAg loss [126, 127]. This is because HBV is still not completely eradicated due to the persistence of cccDNA in the liver and the integration of HBV DNA into host genome even in patients with HBsAg loss or seroconversion. Thus, for patients who stop antiviral therapy based on the endpoint of the HBsAg loss, close monitoring of HBV DNA, ALT levels, and disease progression is extremely necessary.

In addition to HBsAg quantification, other noninvasive serological markers are being developed to guide the antiviral efficacy and the duration or endpoint of therapy: hepatitis B core-related antigen (HBcrAg) and circulating HBV RNA [1].

Both HBcrAg and circulating HBV RNA levels correlate well with the intrahepatic cccDNA levels and may be potential predictive biomarkers to monitor the safe discontinuation of NA therapy [128–130].

3 Developing New Drugs for HBV Treatment

Current antiviral drugs can sufficiently suppress the serum HBV DNA, achieving complete virological response in the majority of CHB patients and thus reducing the morbidity and mortality of HBV-related liver diseases. The combination of Peg-IFN-α with NA either simultaneously or sequentially can enhance the rate of functional cure of CHB (HBsAg loss or seroconversion), but the benefits are mainly limited to a relatively small proportion of patients (≈10%), especially in those with low baseline HBsAg level and on-treatment HBsAg response. The ultimate treatment goal for CHB is to cure HBV infection with the elimination of all forms of potentially replicating HBV, which is hardly achievable with current antiviral therapy due to the persistence of cccDNA (the HBV transcriptional template) in the hepatocyte nucleus [2]. Therefore, novel therapeutic drugs are needed either targeting different steps of HBV life cycle or modulating the host immune system (Fig. 1).

3.1 New Drugs Targeting HBV Life Cycle

3.1.1 HBV Entry Inhibitors

Entry is the first step for HBV infecting hepatocytes. NTCP has been identified as a special functional receptor for HBV entry into hepatocytes [131]. Therefore, entry inhibitors have been proposed as promising agents for protecting uninfected hepatocytes. New drugs targeting viral entry receptor NTCP including Myrcludex B (phase II) and cyclosporin A (in vitro) are being developed and investigated.

Myrcludex B is a synthetic lipopeptide derived from HBV preS1 domain. Binding to NTCP, Myrcludex B not only effectively prevents HBV spread among intrahepatic cells but may also hinder the amplification of intrahepatic cccDNA pool in infected hepatocytes [132]. In a phase I clinical trial, Myrcludex B showed excellent tolerability up to high doses (up to 20 mg intravenously and 10 mg subcutaneously), and the pharmacologic properties followed a two-compartment target-mediated drug disposition model [133]. The results of a phase II clinical trial showed that 10 mg Myrcludex B had more potency of antiviral activity than lower doses, while no noteworthy change of HBsAg concentrations was observed [134]. As Myrcludex B can block the infection of new hepatocytes [132], it may be quite attractive for use in the liver transplantation setting to prevent reinfection of transplanted liver.

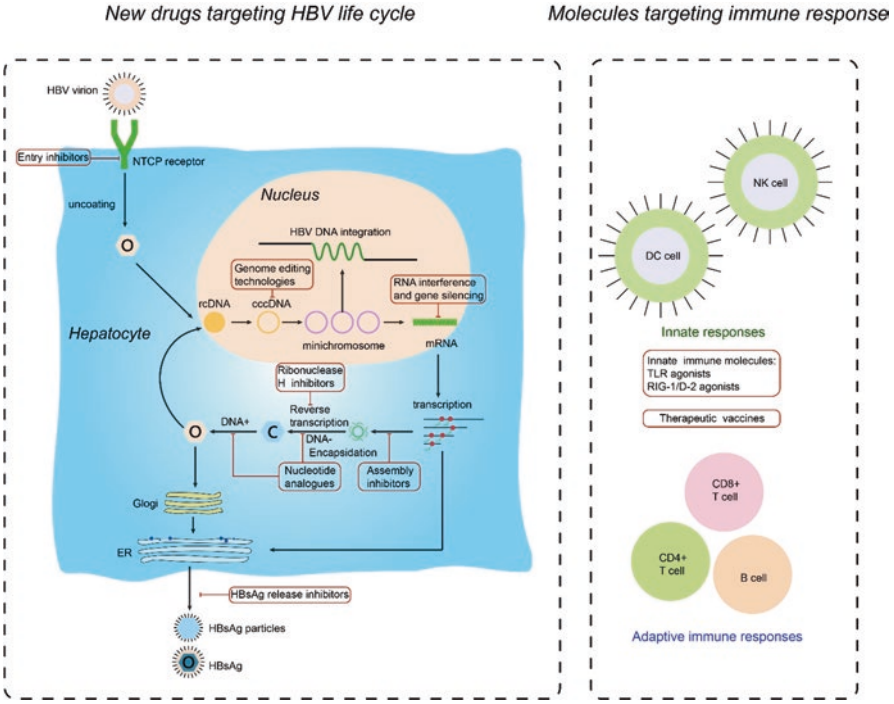

Fig. 1 Development of new anti-HBV drugs either targeting HBV life cycle or modulating host immune response

Left panel: the HBV life cycle including viral entry, trafficking, cccDNA formation, transcription, encapsidation, replication, capsid assembly, and viral secretion is shown. The development of new drugs targeting different steps of HBV life cycle is shown in red box: entry inhibitors such as Myrcludex B, inhibition of cccDNA formation by genome-editing technologies such as TALENs and CRISPR/Cas9, mRNA degradation or translational suppression by RNA interference, assembly inhibitors, ribonuclease H inhibitors, and HBsAg release inhibitors

Right panel: immunomodulators such as TLR agonists, RIG-1/NOD-2 agonists, and therapeutic vaccines can be used to enhance the innate and adaptive immune responses to control HBV infection. Abbreviations: NTCP, sodium taurocholate cotransporting polypeptide; cccDNA, covalently closed circular DNA; rcDNA, relaxed circular DNA; ER, endoplasmic reticulum; TLR, Toll-like receptor; RIG-1, retinoic acid-inducible gene 1; NOD-2, nucleotide-binding oligomerization domain protein 2

Cyclosporin A (CsA), a cyclic non-ribosomal peptide, is usually used as an immunosuppressant in organ transplantation. It has been reported that CsA and its analogues can potentially inhibit the transporter activity of NTCP, thereby blocking HBV entry into hepatocytes [135, 136]. However, it has been found that CsA and its analogues impair sodium-dependent bile acid uptake, thus inducing various adverse events [137]. To identify new compounds that inhibit HBV entry without affecting the NTCP-dependent bile acid uptake, Shimura and his colleagues recently characterized some CsA derivatives and found that SCY450 and SCY995 did not impair

the bile acid uptake and were effective in inhibiting different HBV genotypes and relevant ETV-resistant HBV isolate [138]. Nevertheless, current studies about CsA and its derivatives mostly focus on in vitro experiments; future in vivo studies using animal models and clinical trials are needed.

3.1.2 Therapeutic Approaches Targeting HBV cccDNA

HBV cccDNA is the viral transcription and replication template, and thus its elimination within the hepatocytes is essential for the cure of CHB. Although current anti-HBV therapies such as the use of ETV, TDF, or TAF can potently suppress the HBV DNA to undetectable level, they have little effect on the level and activity of cccDNA within the infected hepatocytes. Therefore, novel therapeutic approaches directly targeting HBV cccDNA are necessary to completely eradicate persistent HBV infections.

APOBEC3 cytidine deaminases are important innate host antiviral factors. It has been reported that the APOBEC3B upregulation triggered by the activation of LTBR can inhibit HBV replication during reverse transcription and degrade cccDNA in the nucleus [139–141]. The inhibition of HBV replication through the activation of the LTBR/APOBEC3 pathway in HBV-infected hepatocytes was recently demonstrated in cell and murine models by using engineered non-lytic T cells expressing HBV-specific T-cell receptors [142]. However, by comparing the intrahepatic cccDNA levels with the expression levels of LTBR and APOBEC3 in the chronically HBV-infected liver biopsy tissues, the activation of the LTBR/APOBEC3 pathway was found to have no major impact on HBV cccDNA metabolism [143]. Future studies are needed to test whether LTBR agonists can degrade cccDNA through the activation of APOBEC3.

Genome-editing technologies including transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system, which are designed to target specific DNA sequences, represent highly promising therapeutic tools to achieve the ultimate goal of curing CHB [144–146]. TALENs comprise a nonspecific Fok1 nuclease domain fused to a customizable sequence-specific DNA-binding domain: transcription activator-like effectors (TALEs) derived from the plant pathogen *Xanthomonas* [147]. The DNA-binding domain can be easily engineered to target and disrupt essentially any specific DNA sequence. It has been reported that TALENs targeting HBV-specific sites within the viral genome led to targeted disruption of approximately 31% of cccDNA in HepG2.2.15 cells and the reduced viral replication in HBV replication murine model without evident toxicity [148]. Despite encouraging results showing the utilization of TALENs against cccDNA, the safe and efficient delivery of the therapeutic transgenes to the infected hepatocytes and the potential off-target effects must be addressed before reaching the clinic. For example, as HBV DNA sequences are frequently integrated into the host genome, what deleterious effects would happen if the designed TALENs cleaved HBV DNA at these sites of HBV integration [149, 150].

CRISPR/Cas system is derived from the acquired immune system of bacteria and archaea against invading foreign DNA via RNA-guided DNA cleavage. This system can be used flexibly by designing sgRNA to any DNA sequences and thus is more easily customizable than TALENs [145]. Recently, several studies have demonstrated that CRISPR/Cas system is able to disrupt or inactivate HBV cccDNA and integrated HBV genomes [151–153]. Li et al. showed that HBV-specific CRISPR/Cas system mediated removal of the full-length integrated HBV DNA and the disruption of HBV cccDNA in a stable HBV cell line [154]. Moreover, recent characterization of smaller *Staphylococcus aureus* Cas9 (SaCas9) has led to the successful package of the derived CRISPR-SaCas9 system into the adeno-associated virus (AAV) type 8 vector [155]. It was shown that the AAV-delivered CRISPR-SaCas9 could efficiently reduce serum HBsAg and HBeAg in HBV transgenic mice during 58-day continuous observation after vein injection [155]. Very recently, Kostyushev et al. showed that CRISPR/Cas9 system from *Streptococcus pyogenes* (*Sp*) and *Streptococcus thermophilus* (*St*) targeting conserved regions of the HBV genome resulted in degradation of over 90% of HBV cccDNA by 6 days post-transfection. Although deep sequencing revealed that *St*-CRISPR/Cas9 had no effect on the host genome, the *Sp*-CRISPR/Cas9 induced off-target mutagenesis [156].

There are challenging issues need to be solved before utilizing CRISPR/cas9 technology to eliminate HBV cccDNA [157]. Firstly, the risk of the intrinsic off-target effects of CRISPR/Cas9 needs to be eliminated. Ideally, the targeted sequences need to be well-conserved among virus isolates and contain nonhomologous sequences in the human genomes, thus avoiding the off-target effects [158, 159]. Secondly, the efficacy of in vivo delivery of the CRISPR/Cas system into hepatocytes needs to be improved. More efficient viral vectors including AAV or nonviral vectors including lipid-like nanoparticles to deliver CRISPR/Cas9 into the hepatocytes need to be explored [160]. Thirdly, reliable and convenient assays for high-throughput quantification of HBV cccDNA are needed. HBV cccDNA levels in HBV-infected hepatocytes are extremely low; although reverse transcription-polymerase chain reaction (RT-PCR) or Southern blot procedures are currently used in basic research studies, these methods are not completely reliable and are also time-consuming and labor-intensive [161–163]. Therefore, a reliable and efficient assay for cccDNA quantification is necessary for the examination of the CRISPR/Cas9 effect on cccDNA.

3.1.3 RNA Interference

RNA interference (RNAi) is a process by which small interfering RNA molecules of 21–25 nucleotides induce gene silencing at the posttranscriptional level to down-regulate the expression of targeted genes. RNAi-mediated inhibition of gene expression and protein production using synthetic small interfering RNAs (siRNAs) has become a tool in antiviral gene therapy [164–166]. In the past, a major barrier for the clinical application of RNAi was the lack of safe and effective delivery vehicle. Recent developments in RNAi technology have overcome the delivery challenge

[166, 167]. The 2018 FDA approval of the first siRNA therapeutic ONPATTRO™ (patisiran) for the treatment of transthyretin-mediated amyloidosis represents an important milestone in the field of RNAi-based therapies.

The potential of RNAi application in CHB treatment has been demonstrated in murine models [168] and infected chimpanzees [169]. Currently, a phase II clinical trial with the RNAi-based agent ARC-520 (developed by Arrowhead Research Corporation) has been conducted in CHB patients [169]. The RNAi ARC-520 was shown to be safe and well-tolerated and significantly decreased HBsAg levels in treatment-naïve HBeAg-positive CHB patients. However, the HBsAg levels were reduced less significantly in HBeAg-negative or long-term NA treatment-experienced CHB patients. This phenomenon is explained by the finding that HBsAg is expressed not only from the episomal cccDNA mini-chromosome but also from transcripts arising from HBV DNA integrated into the host genome, which is the dominant source in HBeAg-negative patients [169], suggesting that ARC-520 only targets the cccDNA-derived pgRNA rather than the integrated HBV DNA. To overcome the limited efficacy of ARC-520 in HBeAg-negative patients, ARO-HBV (JNJ-3839, targeting two sources of HBsAg) has been developed and assessed by Arrowhead [170]. The results obtained from a phase II trial were announced at the 2019 annual AASLD meeting that monthly usage of ARO-HBV could effectively reduce all viral products, including HBV DNA, HBV RNA, HBeAg, HBcrAg, and HBsAg [171].

AB-729 developed by Arbutus is another RNAi therapeutic targeted to hepatocyte using a novel conjugated N-acetylgalactosamine (GalNAc) delivery technology. It acts on all HBV RNA transcripts, enabling the suppression of all viral antigens, including HBsAg. Preclinical study has demonstrated the anti-HBV activity of AB-729 in vitro [172]. AB-729 phase Ia/Ib clinical trial (AB-729-001) is a single- and multiple-dose clinical trial to investigate the safety, tolerance, pharmacokinetics, and pharmacodynamics of AB-729 subcutaneously administered to healthy subjects and CHB patients.

In addition to the abovementioned RNAi molecules, other RNAi-based agents currently under development are summarized in Table 5.

3.1.4 Capsid Assembly Inhibitors/Modulators

HBV capsid has multiple functions in HBV life cycle, including genome packaging, reverse transcription, and intracellular trafficking, making it an excellent target for development of new antiviral therapy [173]. Small-molecule compounds targeting core protein or capsid, also termed as core assembly modulators (CAMs), can interfere with pgRNA encapsidation, HBV DNA replication by misdirecting or accelerating the formation of capsid-like structures [174]. Based on their different effects on the capsid assembly, CAMs can be categorized into two classes: the type I CAMs, represented by heteroaryldihydropyrimidine (HAP), function to misdirect the formation of aberrant or non-capsid structures; the type II CAMs, represented

Table 5 Developing new drugs for HBV treatment

Developing new drugs for HBV treatment	Category	Mechanism of action	Compound	Development status
New drugs targeting HBV life cycle	Entry inhibitors	Targeting viral entry receptor NTCP	Myrcludex B	II
			Cyclosporin A	Preclinical
	Therapeutic approaches targeting HBV cccDNA	Knockout of HBV cccDNA	ZFNs and TALENs	Preclinical
	RNA interference	Targeting viral sequences and inducing mRNA degradation or translational suppression	ARC-520	II
			ARO-HBV	I/II
			RG6004	I/II
			AB-729	I
			Vir-2218 (ALN-HBV)	Preclinical
			BB-103	Preclinical
			Lunar-HBV	Preclinical
	Capsid assembly inhibitor/ modulator	Inhibiting HBV replication by causing destabilization of viral nucleocapsid	GLS4	II
			JNJ-6379	III
			NVR 3-778	IIa
			ABI-H0731	II
			Bay 41-4109	I
			AB-506	I
			ABI-H2158	I
			RG7907	I
			QL-007	I
			GLP-26	Preclinical
			CB-HBV-001	Preclinical
			ABI-H3773	Preclinical
	New nucleoside/ nucleotide analogues	New nucleoside/ nucleotide analogues	Besifovir	III
			CMX157	IIa
	Ribonuclease H inhibitors	Blocking the production of the plus-polarity DNA strand and leading to the section of biologically inert viral genomes	α-Hydroxytropolones	Preclinical
			N-Hydroxyisoquinolinediones	Preclinical
			N-Hydroxypyridinediones	Preclinical
	HBsAg release inhibitors	Inhibiting the release of HBsAg	REP 2139	II
			REP 2165	II

(continued)

Table 5 (continued)

Developing new drugs for HBV treatment	Category	Mechanism of action	Compound	Development status
Molecules targeting immune response	TLR-7 and TLR-8 agonists	Leading to the production of endogenous cytokines such as IFN to control the virus	RO7020531	II
			GS-9688	II
			GS-9620	Preclinical
	RIG-1/NOD-2 agonists	Eliminate infected hepatocytes, help noninfected hepatocytes establish an antiviral state	SB 9200	II
	Programmed death-1 inhibitors	Blockade PD-1 pathway, promote the proliferation of HBV-specific T cells	PD-1 inhibitors	II
Therapeutic vaccine	Therapeutic vaccine	Stimulating CD_4 and CD_8 T-cell response	HBsAg-HBIG complexes	III
			INO-1800	I
			HB-110	I
			GS-4774	II
			TG-1050	I
			AIC 649	I
			HeP T cell	I

by phenylpropenamides (PPAs) and sulfamoylbenzamides (SBAs), function to accelerate the formation of morphologically intact empty capsids [175, 176].

Antiviral profiling study with BAY41-4109 (HAP) and JNJ-632 (SBA) in primary human hepatocytes has revealed that CAMs not only inhibit HBV replication but also HBV RNA transcription and antigen production, suggesting that CAMs have a dual mechanism of action, inhibiting early and late steps of the viral life cycle [177, 178]. A very recent antiviral profiling study with another CAM molecule NVR 3-778 (SBA) also showed potent anti-HBV activity with a mean EC_{50} of 0.40 μM in HepG2.2.15 cells, and the combination of NVR 3-778 with NAs in vitro resulted in synergistic antiviral activity [174]. Similarly, the potent anti-HBV activity of NVR 3-778 was also shown in a mouse model with HBV-infected humanized liver [179]. NVR 3-778 and Peg-IFN-α in combination showed higher antiviral activity than each compound alone or ETV in the mouse model [179]. In a phase I

clinical study, the combination of NVR 3-778 with Peg-IFN-α led to more reduction of HBV DNA and RNA than monotherapy in HBeAg-positive CHB patients [180]. NVR 3-778 is now in phase II clinical trial.

Other potent CAMs that have also proceeded to clinical trials include JNJ-6379 (SBA) in phase III [181], GLS4 (HAP) in phase II [182], and ABI-H0731 (the first-generation core protein allosteric modifier, CpAM) in phase II (Table 5) [183, 184]. Very recently, through high-throughput screening of an Asinex small-molecule library containing approximately 20,000 compounds, 8 novel structurally distinct CAMs including BA-53038B have been identified [185].

3.1.5 New Nucleoside/Nucleotide Analogues

Current highly potent and low-resistant NAs (ETV, TDF, and TAF) can effectively suppress the serum HBV DNA to undetectable level in the majority of CHB patients. However, if a new NA with even greater inhibition of intrahepatic DNA replication can be developed, it may provide rescue therapy to CHB patients with poor response or drug resistance to current first-line NAs. Several new NAs are currently under different clinical phases of development.

Besifovir (LB80380/BSV), a novel acyclic nucleotide phosphonate with a similar chemical structure to tenofovir, was approved by the Korean Ministry of Food and Drug Safety in 2017 for CHB treatment. The antiviral efficacy of BSV was demonstrated to be similar with that of ETV in a phase IIb multicenter randomized trial [186]. However, the side effect of L-carnitine depletion occurred in 94.1% (64/75) patients receiving BSV treatment [186]. Recently, a phase III clinical trial was conducted in Korea to compare the antiviral efficacy and safety of BSV and TDF in 197 CHB patients [187]. Patients were randomly assigned to groups receiving BSV (150 mg, n = 99) or TDF (300 mg, n = 98) for 48 weeks. After 48 weeks, BSV group continued BSV treatment, whereas the TDF group switched to BSV treatment for an additional 48 weeks. The results showed that at week 48, the rates of virologic responses (HBV DNA <69 IU/mL or 400 copies/ml) were 80.9% and 84.9% in the BSV group and TDF group, respectively. At week 96, 87.2% of patients in the BSV-BSV and 85.7% of patients in the TDF-BSV group achieved virologic response. Thus, the 1- and 2-year treatment outcome of CHB patients with BSV was comparable to that of TDF. There were no BSV-related drug resistance mutations, osteoporosis, or renal toxicity. However, to secure a niche in the field of anti-HBV medicine, the safety and efficacy of BSV from long-term follow-up study is needed [187].

Another new NA CMX157 is currently under phase II clinical trial [184, 188] (Table 5).

3.1.6 Ribonuclease H Inhibitors

In HBV life cycle, the viral pgRNA is encapsidated by the core antigen and is reverse-transcribed by the viral polymerase to minus-strand DNA. During the minus-strand DNA elongation, the viral pgRNA is degraded by ribonuclease H

(RNaseH) to permit the synthesis of plus-strand DNA [189]. Mature capsid particles are either secreted from the cell as virions or cycled back to the nucleus to amplify and/or replenish the cccDNA pool. Inhibitors targeting the RNaseH activity would truncate the minus-strand DNA and block the synthesis of plus-strand DNA, thus blocking the release of infectious virions and the amplification and/or replenishment of the cccDNA pool [190]. Recent low-throughput anti-HBV RNaseH screening pipeline has led to the identification of several chemical classes of potential HBV RNaseH inhibitors including α-hydroxytropolones (α-HT), *N*-hydroxyisoquinolinediones (HID), and N-hydroxypyridinediones (HPD) [191, 192]. These RNaseH inhibitors are promising candidates for developing new anti-HBV drugs and could be used in combination with existing anti-HBV drugs or other novel antivirals under development to improve the functional cure of CHB in the future.

3.1.7 HBsAg Release Inhibitors

HBsAg, the most abundant circulating viral antigen, has been reported to contribute to T-cell tolerance and exhaustion, leading to the attenuation of host immune response [167]. Thus, inhibition of HBsAg release may help to restore the HBV-specific T-cell-mediated immune response. Nucleic acid polymers (NAPs), the phosphorothioated oligonucleotides, have been shown to block the assembly of subviral particles (the primary source of circulating HBsAg), thus inhibiting the release of HBsAg from infected hepatocytes [173, 193]. Recent clinical studies have demonstrated that treatment with the NAPs REP 2139 or its analogue REP 2165 leads to the loss of HBsAg in the majority of CHB patients, regardless of HBeAg status or the presence of HDV coinfection [194–197]. In the open-label, nonrandomized, phase 2 clinical study (REP 301 study), 12 HBeAg-negative, HBV-/HDV-coinfected patients treated with REP 2139-Ca in combination with Peg-IFN-α resulted in 5 patients achieving HBsAg loss and 7 patients achieving HDV RNA negative 1 year after the termination of treatment [194]. Very recently, the final follow-up data from the REP 401 study aiming to assess the safety and efficacy of REP 2139-Mg or REP 2165-Mg (250 mg iv qW) in combination with TDF (300 mg PO qD) and Peg-IFN-α (180 μg SC qW) in 40 HBeAg-negative CHB patients were reported by Replicor Inc. (http://replicor.com/, as of August 2019). The data showed that 40% of participants achieved functional cure of HBV. Meta-analysis of all HBeAg-negative patients in the REP 301 and the REP 401 studies revealed that the extent of transaminase flare activity correlated with significant HBsAg reductions from baseline and greater chance of achieving functional cure. Transitioning REP 2139-Mg from IV to SC administration is expected to improve tolerability, thus enabling higher frequency of administration and further improvement of HBsAg loss. The safety and efficacy of 48 weeks of subcutaneously administered REP 2139-Mg in combination with TDF and Peg-IFN-α against HBV and HDV infections is planned to be assessed in upcoming REP 501 trial (http://replicor.com/, as of August 2019).

3.2 New Agents Modulating Host Immune Response

Host immune responses including innate and adaptive immune response play important roles in the control of chronic HBV infection. Based on the immunopathogenesis of HBV infection, modulating innate or adaptive immunity or both in combination with other direct antiviral drugs to control HBV infection may provide new strategies for CHB treatment. The immunomodulating therapeutic agents under development include TLR-7 and TLR-8 agonists, retinoic acid-inducible gene 1 (RIG-I)/nucleotide-binding oligomerization domain protein 2 (NOD-2) agonists, and programmed death-1 (PD-1) inhibitors, therapeutic vaccines, and others.

3.2.1 TLR-7 and TLR-8 Agonists

Toll-like receptors serve as the first-line defense against invading pathogens [198]. Activation of TLR may help to fight against HBV. TLR agonists (TLR-7 and TLR-8) can induce endogenous interferon production and innate responses, leading to induction of ISGs and other signaling cascades to inhibit HBV replication [199–201]. Currently, the TLR-7 agonists (GS9620, RO7020531) and TLR-8 agonist (GS-9688) are under different phases of clinical trials.

The TLR-7 agonist GS-9620 was shown to induce sustained reduction of HBV viral load and serum HBsAg levels mainly via a type I IFN-α-dependent mechanism in the human hepatocytes [202] and woodchuck [203] and chimpanzee models of CHB [204]. However, in a recent Italian study enrolling 28 CHB patients with HBV suppression (tested negative for HBeAg) by NA therapy, 12-week administration of different doses of GS-9620 (oral, once weekly) appeared to increase T-cell and NK-cell responses, but had no significant effect on HBsAg levels in the enrolled CHB patients [205]. Similarly, in a phase II, double-blind, randomized, placebo-controlled study enrolling 162 CHB patients who are virally suppressed by NA treatment, the administration of GS-9620 resulted in dose-dependent pharmacodynamic induction of ISG15, but had no significant effect on the systemic induction of IFN-α expression and no significant effect on the levels of HBsAg [206]. RO7020531, another TLR-7 agonist, when used in combination with a capsid assembly modulator RO7049389, was reported to significantly reduce the levels of HBV DNA and HBsAg in HBV replication mouse model [207]. At the 2019 APASL annual meeting, RO7020531 was reported to be safe and well-tolerated in healthy volunteers and could induce the increase of IFN-α-related cytokine and ISGs [208]. Further evaluation of the TLR-7 agonist in combination with anti-HBV drugs on the reduction of HBV replication, HBsAg, and cccDNA levels in treating CHB patients is needed.

The TLR-8 agonist GS-9688 was shown to induce sustained efficacy and HBsAg serological conversion in the CHB woodchuck model [209]. A randomized, blind, placebo-controlled study showed that GS-9688 was well-tolerated in CHB patients [210]. Currently, GS-9688 is undergoing phase II clinical trials.

3.2.2 RIG-I/NOD-2 Agonists

RIG-I and NOD-2 are the pattern-recognition receptors recognizing signature patterns of foreign RNA, resulting in the activation of the IFN-α signaling pathway and the subsequent induction of ISGs and pro-inflammatory cytokines [211, 212]. Recently, it was reported that the 5′-ε region of HBV-derived pgRNA is recognized by RIG-I, resulting in the predominant production of type III, but not type I, IFNs in human primary hepatocytes. In addition, the RIG-I was also found to counteract the interaction of HBV polymerase with the 5′-ε region of pgRNA in an RNA-binding dependent manner, resulting in the suppression of HBV replication [212].

The RIG-I agonist SB 9200 (inarigivir) is a novel oral modulator of innate immunity [213]. Inarigivir has been investigated in a phase II multicenter clinical trial (the ACHIEVE study) enrolling 80 treatment-naïve non-cirrhotic CHB patients. The enrolled patients were randomized 4:1 to receive ascending doses of inarigivir (25 mg, 50 mg, 100 mg, and 200 mg) or placebo for 12 weeks, followed by a switch to 300 mg TDF daily for a further 12 weeks. At the 2018 APASL annual meeting, the first cohort inarigivir 25 mg was reported to have good safety and significant antiviral effects on HBV replication [214]. At the 2019 EASL annual meeting, the final results of the ACHIEVE study were reported [215]. It was demonstrated that the reductions of HBV DNA and HBV RNA were observed in both HBeAg-positive and HBeAg-negative patients in a dose-dependent manner, while the extent of HBV RNA reduction was greater in HBeAg-negative patients. The HBsAg response (defined as >0.5 $\log_{10}$ reduction at either 12 or 24 weeks) was seen in 22% of patients, but the HBsAg decline was not dose-dependent. Further investigations of inarigivir at doses of up to 400mg daily in combination with TDF or added to NA-suppressed CHB patients are underway [215].

3.2.3 Programmed Death-1 (PD-1) Inhibitors

Adaptive immune responses are essential in obtaining successful control of viral infection, and T-cell responses are the important contributors [216]. However, virus-specific T cells are exhausted in chronic HBV infection, which is one of the major barriers to eliminating the virus. In the course of chronic HBV infection, overexpressed inhibitory receptors on T cells are associated with dysfunctional T-cell responses. Programmed death receptor 1 (PD-1), the most highly expressed inhibitory receptor on HBV-specific T cells, together with the increased expression of PD-L1 (PD-1's ligand) in HBV-infected hepatocytes likely contributes to the exhaustion of T cells and the high HBV replication levels in CHB patients [217–220]. Thus, blockade of PD-1/PD-L1 pathway would promote the proliferation of HBV-specific T cells, thus restoring the function of T cells to control HBV [217, 221].

Reversing "T-cell exhaustion" through the blockade of the PD-1/PD-L1 pathway to improve specific anti-HBV T-cell responses has been proven in in vitro, HBV mouse and woodchuck models [222, 223]. Very recently, the safety and immunologic efficacy of the PD-1 inhibitor nivolumab were investigated in a phase Ib study

enrolling 24 NA-suppressed HBeAg-negative CHB patients [224]. The enrolled patients were treated with either single dose of nivolumab at 0.1 mg/kg (n = 2) or 0.3 mg/kg (n = 12) or two doses of nivolumab 0.3 mg/kg (at baseline and at week 4) in combination with 40 yeast units of the HBV therapeutic vaccine GS-4774 (n = 10). Twelve weeks after the administration of nivolumab, no significant reduction of HBsAg level was observed in the two patients receiving nivolumab at 0.1 mg/kg. Of the 22 patients who received 0.3 mg/kg nivolumab with or without GS-4774, 20 (91%) had significant HBsAg decline from baseline. One out of the ten patients receiving the combination of nivolumab plus GS-4774 achieved sustained HBsAg loss. Thus, this pilot study supports the inclusion of PD-1/PD-L1 blockade in future combination strategies toward functional cure of chronic HBV infection [224].

In addition to above mentioned immunomodulators, other kinds of immunomodulators including IFN-λ [225], IL-12 [226–228], IL-21 [229, 230], and IL-8 [231] have also been reported to play a role in anti-HBV immunity.

3.2.4 Therapeutic Vaccines

Since the naturally cured HBV infection is accompanied by immune reconstitution [232], stimulation of HBV-specific B- and T-cell immunity by therapeutic vaccination represents a potential approach to overcome the immune tolerance in CHB patients [232]. Different categories of therapeutic vaccines (including protein-based, DNA-based, and vector-based vaccines) have been developed and investigated in both animal models and humans [233, 234]. Of note, most of the therapeutic vaccines in clinical trials are being investigated in combination with current antiviral drugs.

Protein-Based Vaccines

Protein vaccines include subunit vaccines and antigen-antibody complex vaccines. In an open-label and controlled clinical study, 195 HBeAg-positive CHB patients were randomized to receive 12 doses of AS02B-adjuvanted HBsAg vaccine plus LAM daily or LAM daily alone for 52 weeks [235]. However, disappointingly, the combined administration of vaccine and LAM did not demonstrate superior clinical efficacy in HBeAg-positive CHB patients as compared to LAM therapy alone. To increase the antigen-based immune therapy for CHB, a vaccine formulation containing both HBsAg and HBcAg (designated as HeberNasvac) was developed [236]. In a recent open-label phase III study, 160 CHB patients were randomized 1:1 to receive 10 doses of HeberNasvac (100 μg HBsAg and 100 μg HBcAg per dose) via nasal spray or 48 subcutaneous injections of Peg-IFN-α (180 μg per dose) [237]. The HBeAg loss was found to be more frequent in the HeberNasvac group as compared to the Peg-IFN-α group, but the antiviral effect was comparable in the two groups.

The antigen-antibody (HBsAg-HBIG) complex therapeutic vaccine with alum as adjuvant showed promising results in a double-blind, placebo-controlled, phase IIb

clinical trial [238], but results from a phase III clinical trial enrolling 450 CHB patients treated with alum-adjuvanted HBsAg-HBIG or alum alone were disappointing as no significant difference in reduction of HBV DNA level and normalization of liver function was observed in the two groups [239]. The unfavorable results from the therapeutic protein vaccines are most likely due to the fact that those vaccines preferentially induce antibody but not cytotoxic T-cell responses [233].

DNA-Based Vaccines

DNA-based vaccines encoding HBV envelope proteins were designed to induce HBV-specific T cells. INO-1800, a multi-antigen DNA vaccine encoding HBsAg and a consensus sequence of HBcAg, is now in phase I clinical trial administered with or without INO-9112 encoding human IL-12 in 90 CHB patients either on ETV or TDF treatment [240]. HB-110, another multi-antigen DNA vaccine encoding HBsAg, PreS1Ag, HBcAg, HBV polymerase, and human IL-12, was recently investigated in a phase I clinical trial [241]. The enrolled 27 ADV-treated CHB patients were randomized to receive a combination of HB-110 plus ADV or ADV alone. The results showed that the HB-110 add-on to ADV did not show greater T-cell responses and HBeAg loss than ADV alone [241]. Both INO-1800 and HB-110 vaccines need to be administered via in vivo electroporation to enhance vaccine antigen expression and immunogenicity, and the purpose of adding IL-12 as an adjuvant was aimed to rescue the antiviral function of exhausted HBV-specific T cells.

Vector-Based Vaccines

The currently studied vector-based therapeutic vaccines mainly include the yeast-based vaccine GS-4774 [242, 243] and the adenovirus-based vaccine TG1050 [244, 245].

GS-4774 is a recombinant, heat-killed, *Saccharomyces cerevisiae* yeast-based vaccine expressing HBV-specific antigens including HBsAg, HBcAg, and HBx. In a randomized, open-label, phase II study of GS-4774, 178 non-cirrhotic CHB patients who were virally suppressed by NAs were randomized 1:2:2:2 to receive NA alone or NA plus GS-4774 at the doses of 2, 10, or 40 yeast units subcutaneously every 4 weeks until week 20 [243]. It was shown that GS-4774 was safe and well-tolerated but did not provide significant reductions in serum HBsAg in those treatment-experienced CHB patients. In another phase II study, the safety and efficacy of GS-4774 alone or in combination with TDF were investigated in 195 treatment-naïve CHB patients [242]. Although GS-4774 in combination with TDF was able to induce a strong immunomodulatory effect (the increased production of IFN-γ, TNF-α, and IL-2 by CD8+ T cell), it did not reduce the HBsAg levels in those treatment-naïve CHB patients, either, suggesting that GS-4774 might be used in combination with other antiviral agents to boost the anti-HBV immune response [242].

TG1050 consists of a non-replicative adenovirus 5 vector encoding a unique large fusion protein composed of a modified HBV core, a modified HBV polymerase, and selected domains of the envelope proteins [193]. Injection of TG1050 was able to induce a robust T-cell response and to exert an antiviral effect in HBV-naïve and HBV-persistent mouse models [244]. The safety and efficacy of TG1050 in CHB patients under NAs were assessed in a phase Ib clinical trial [245]. TG1050 was found to have a good safety profile and capable of inducing HBV-specific cellular immune response. Further clinical evaluation of TG1050 in combination with other anti-HBV agents is needed [245].

4 Conclusions

In conclusion, over the past 20 years, tremendous progress has been made in the understanding of HBV pathogenesis and the treatment of CHB. The advent of NAs has made CHB an easily treatable disease. The current anti-HBV therapy with potent and high genetic barrier NAs (ETV, TDF, and TAF) can suppress the viral replication to undetectable level in the majority of CHB patients, preventing the progression of CHB to cirrhosis and markedly decreasing the rates of HBV-related HCC. The combination of NAs and Peg-IFN-α even makes the functional cure of CHB possible in selected patients with low baseline HBsAg level and on-treatment HBsAg response to Peg-IFN-α. To increase the rate of functional cure of CHB, a combination of the existing anti-HBV drugs and one or more of the abovementioned new antiviral agents, either the direct antiviral drugs targeting the different steps of HBV life cycle or the indirect antiviral drugs modulating the host immune responses, will be necessary. With the concerted efforts of basic research scientists and clinical experts from both professional societies and pharmaceutical companies, the ultimate eradication of HBV infection is likely to be achieved in the foreseeable future.

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