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Virological Basis for the Cure of Chronic Hepatitis B

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Abstract

Hepatitis B virus (HBV) has infected one-third of world population and 240 million people are chronic carriers, to whom a curative therapy is still not available. Similar to other viruses, persistent HBV infection relies on the virus to exploit host cell functions to support its replication and efficiently evade host innate and adaptive antiviral immunity. Understanding HBV replication and concomitant host cell interactions is thus instrumental for development of therapeutics to disrupt the virus-host interactions critical for its persistence and cure chronic hepatitis B. Although the currently available cell culture systems of HBV infection are refractory to genome-wide high throughput screening of key host cellular factors essential for and/or regulating HBV replication, classic one-gene (or pathway)-at-a-time studies in last several decades have already revealed many aspects of HBV-host interactions. An overview of the landscape of HBV-hepatocyte interaction indicates that in addition to more tightly suppressing viral replication by directly targeting viral proteins, disruption of key viral-host cell interactions to eliminate or inactivate the covalently closed circular (ccc) DNA, the most stable HBV replication intermediate that exists as an episomal minichromosome in the nucleus of infected hepatocyte, is essential to achieve a functional cure of chronic hepatitis B. Moreover, therapeutic targeting of integrated HBV DNA and their transcripts may also be required to induce HBsAg seroclearance and prevent liver carcinogenesis.

As obligate intracellular parasites, successful colonization of cells by viruses depends on strategies evolved to exploit cellular functions to support their replication and capability to overcome cellular antiviral defenses. It is the dynamic virus-host interaction that determines the pathogenesis and outcomes of a viral infection. Therefore, identification of host cellular factors that support or restrict viral replication and elucidation of the underlying mechanisms are important to understand viral pathogenesis and develop antiviral therapeutics to treat viral diseases[1]. The advent of omics technologies in the last decades allows for investigation

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of virus-host interaction at the levels of genome, transcriptome, proteome and metabolome and identification of host cellular proviral and restriction factors through genome-wide genetic screenings[2]. These new technologies have profoundly shaped the landscape of virus-cell interaction research and revealed potential molecular targets for development of antiviral therapeutics[3].

Hepatitis B virus (HBV) is a member of *Hepadnaviridae* family and contains a 3.2 kb, partially double-stranded, relaxed circular (rc) DNA genome[4]. Hepatocytes are the primary host cells of HBV. As depicted in Fig. 1, HBV infection begins by binding to its cellular receptor, sodium taurocholate cotransporting polypeptide (NTCP), on the surface of hepatocytes and delivering viral nucleocapsids into the cytoplasm *via* endocytosis. The viral rcDNA genome in nucleocapsid is then transported into the nucleus and converted into episomal covalently closed circular (ccc) DNA to serve as a template for transcription of viral RNA. Unlike other DNA viruses, HBV replicates its genomic DNA *via* viral DNA polymerase self-primed reverse transcription of a RNA pregenome in the cytoplasmic nucleocapsid. Briefly, binding of viral DNA polymerase to the stem-loop structure at the 5' terminus of pregenomic (pg) RNA initiates their packaging by 120 copies of core protein dimers to form a nucleocapsid. Inside the nucleocapsid, viral DNA polymerase converts the pgRNA first to a single-stranded DNA and then to rcDNA. The rcDNA-containing mature nucleocapsids can either acquire an envelope and be secreted out of cells as infectious virions or deliver the rcDNA into the nucleus to amplify cccDNA pool, the sole transcription template to support HBV replication[5].

While vast majority of adulthood HBV infection is resolved within six months by host antiviral immune responses, particularly the vigorous polyclonal cytotoxic T lymphocyte response against multiple viral proteins, infection at early childhood usually fails to induce a sufficient immune response to clear the virus and results in a life-long persistent infection[6]. Chronic HBV infection is associated with high risk of severe liver diseases, including cirrhosis, hepatocellular carcinoma (HCC) and liver failure[7]. The currently available antiviral therapeutics, including alpha-interferon (IFN-α) that regulates host antiviral immune response and six nucleos(t)ide analogues (NUCs) that inhibit HBV DNA polymerase, can potently suppress viral replication and prevent the progression of liver diseases, but fail to cure the viral infection[8]. This is due primarily to their inability to eliminate cccDNA and restore the dysfunctional antiviral immune response against HBV[5, 9]. Investigation of HBV-host cell interaction will reveal cellular and viral functions that are essential for the stable maintenance and proper function of cccDNA to support the persistent infection and can thus be pharmacologically targeted for elimination or functional inactivation of cccDNA and ultimate cure of chronic hepatitis B. Although the currently available cell culture systems supporting HBV infection are not suitable for genome-wide high throughput screening of key host cellular factors essential for and/or regulating HBV replication, studies of HBV virology in the last several decades have already revealed many aspects of HBV-hepatocyte interaction. Integration of the existing knowledge should serve as a starting point toward construction of a comprehensive molecular network of HBV-hepatocyte interaction and establishment of a knowledge base for continuing search and validation of therapeutic targets for the cure of chronic HBV infection.

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1. HBV replication depends on and is regulated by hundreds of host cellular proteins

Although numerous studies have shown that HBV does not induce an overt pattern recognition receptor-mediated innate immune response in infected hepatocytes[10], classical virology studies have already revealed a very comprehensive molecular landscape of HBV-hepatocyte interaction and identified hundreds of host cellular genes that are required for, or regulate, HBV infection of hepatocytes at every step of the viral life cycle.

Entry: delivery of nucleocapsids into the cytoplasm

Three cellular proteins, heparan sulfate proteoglycan (HSPG), sodium taurocholate cotransporting polypeptide (NTCP, encoded by SLC10A1) and glypican 5, have been identified as attachment factor, receptor and co-receptor for the infectious entry of HBV into hepatocytes, respectively. The large envelope protein (LHBs) on the surface of HBV virions interacts with HSPG to attach virion particles on the surface of hepatocytes[11]. The myristoylated N-terminal preS1 domain of LHBs subsequently binds NTCP, *bona fide* receptor for HBV[12]. HBV entry can be efficiently blocked, *in vitro* and *in vivo*, by Myrcludex B, a synthetic N-acylated preS1 lipopeptide[13]. In spite of its requirement for HBV infection of HepG2-NTCP cells, the mechanism of glypican 5 in HBV entry remains elusive[14]. Moreover, whether HBV is internalized into hepatocytes *via* clathrin-mediated or caveolin-dependent endocytosis remains controversial[15]. Following endocytosis, HBV must travel through the complex network of the endocytic pathway and release nucleocapsids. While the dependence of HBV infection on Rab5 and Rab7 expression suggests that HBV transport from early to mature endosomes is required for infectious entry, where and how the viral envelope-host cellular membrane fusion and release of nucleocapsid into the cytoplasm take place remain to be determined[16].

Nucleocapsid transport and viral genomic DNA nuclear import

Upon reaching the cytoplasm, nucleocapsids have to be transported through the viscous cytoplasm to nuclear pore complexes (NPCs). Nucleocapsids disassemble at the basket of NPCs and viral genomic DNA are released into the karyoplasm where it is converted into cccDNA by cellular DNA repair machinery[17]. As depicted in Fig. 1, in addition to the incoming virion DNA, cccDNA can also be synthesized from intracellular progeny nucleocapsid DNA[18]. However, although HBV cccDNA intracellular amplification pathway works efficiently in hepatoma cell lines supporting HBV replication[19], it is less efficient in HBV-infected hepatoma cells or primary human hepatocytes[17, 20]. Mechanism of this discrepancy remains to be investigated. Regardless of their origins, the intra-cytoplasmic transportation and viral genomic DNA nuclear import of nucleocapsids ought to take place in a coordinated manner. *First*, a recent study showed that both double-stranded DNA-containing mature nucleocapsids and empty capsids, but not pgRNA-containing capsids, can be actively transported through the cytoplasm towards the nucleus[21]. It was further demonstrated that dynein light chain LL1 functions as an interaction partner linking capsids to the dynein motor complex and retrograde transport capsids along microtubules towards the NPCs[21]. *Second*, carboxyl terminal domain (CTD) of core protein contains two

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overlapping bipartite nuclear localization signals (NLS) and their exposure on the exterior of capsids is modulated by genomic DNA maturation or CTD phosphorylation. The exposed NLS interacts with nuclear import receptors and initiates nuclear transport of capsids[22]. Interestingly, while empty capsids interact with the nuclear import receptor importin-β directly, mature nucleocapsids need the adaptor molecule importin-α, most likely due to the differences in exposure of core protein CTD and/or its phosphorylation statu[22a, 22b, 23]. *Third*, premature or enhanced nucleocapsid uncoating (or disassembly) induced by core protein allosteric modulator (CpAM) treatment or core protein mutations results in reduced amounts of cytoplasmic mature HBV double-stranded DNA, but increased amounts of nuclear deproteinized (DP)-rcDNA and cccDNA[19a, 24]. A plausible explanation is that the premature/enhanced nucleocapsid uncoating increases core protein CTD exposure and the nucleocapsids are thus imported into nuclei *via* the more efficient importin-β pathway. This hypothesis is currently under investigation in our laboratory. *Finally*, NPCs are composed of ~30 different proteins collectively called nucleoporins (Nups)[25]. Interaction with the NLS on capsids changes the structure of import receptors, allowing interaction with Nup358 at the cytoplasmic face of NPC. The capsid is then translocated through the nuclear pore and disassociates from importins in the nuclear basket and directly binds to Nup153, followed by capsid disassembly and diffusion of rcDNA into the karyoplasm[26].

Conversion of viral genomic DNA into cccDNA

It is generally acknowledged that the conversion of viral genomic DNA to cccDNA takes place in the nucleus and is catalyzed by host cellular DNA repair proteins. The strict requirement of Ku-80 and ligase IV strongly suggests that formation of cccDNA from double-stranded linear (dsl) DNA, a minor species of HBV genome derived from *in situ* priming of positive strand DNA, is through non-homologous end joining (NHEJ) DNA repair pathway[27]. However, only a few biochemical reactions essential for cccDNA synthesis from rcDNA precursor have been investigated. *First*, removal of viral DNA polymerase covalently linked to the 5' terminus of rcDNA minus strand is an essential step to convert rcDNA into cccDNA. Tyrosol-DNA phosphodiesterase 2 (TDP2), an enzyme responsible for resolving topoisomerase II-DNA adduct[28], has been demonstrated to cleave the tyrosyl-minus strand DNA linkage *in vitro*[29]. However, TDP2 gene knockout did not inhibit cccDNA formation during HBV infection of NTCP-expressing HepG2 cells[30] and only delayed intracellular amplification of duck hepatitis virus (DHBV) cccDNA[29], suggesting that additional cellular function(s) can process the 5' terminus of rcDNA minus strand. *Second*, failure of viral DNA polymerase inhibitors to block cccDNA formation during *de novo* HBV infection of hepatocytes suggests that the completion of positive strand of rcDNA from viral particles is not catalyzed by viral DNA polymerase, but cellular DNA polymerase(s). A genetic screening in HBV infection of NTCP-expressing HepG2 cells identified cellular DNA polymerase ĸ, and to a lesser extent, DNA polymerase λ, are required for *de novo* cccDNA synthesis[20]. DNA polymerases ĸ and λ play a role in translesion DNA synthesis to bypass base damage in DNA and NHEJ DNA repair pathway, respectively. Exact biochemical reactions catalyzed by those cellular DNA polymerases, involving the elongation of positive strand DNA or filling the gaps in positive and negative strands after end processing, remain to be determined. It is also interesting to know how those error-prone DNA polymerases perfectly repair rcDNA to cccDNA. *Third*, it is obvious

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that ligation of both strands of viral DNA is essential for cccDNA synthesis. Recent identification of rcDNA species with covalently closed negative strand in hepatoma cells supporting HBV DNA replication suggests that this negative-strand closed rcDNA is possibly the intermediate of cccDNA formation from rcDNA precursor and argues that repairing of negative and positive strands of rcDNA sequentially takes place[31]. Moreover, a recent genetic study demonstrated that knocking out either DNA ligase I or ligase III significantly compromised HBV and DHBV cccDNA synthesis from *de novo* infection and intracellular amplification pathways[27b], suggesting that both the ligases are required for cccDNA synthesis from rcDNA. Finally, it was reported recently that activation of retinoid X receptor alpha (RXRα) inhibited *de novo* HBV cccDNA synthesis in HBV infected human hepatocytes through inducing expression of the genes in arachidonic acid/eicosanoids biosynthesis pathways and production of arachidonic acid[32]. Obviously, further investigation is required to determine the biochemical reaction in cccDNA synthesis that is inhibited by this cellular metabolism pathway.

cccDNA minichromosome and transcription regulation

As depicted in Fig. 2, expression of the four open reading frames (ORFs) of HBV genome are regulated by four promoters (basal core, preS1, preS2/S, and X) and two enhancers (Enh I and Enh II) to transcribe four major species (3.5, 2.4, 2.1 and 0.7 kb) of mRNA. While 2.4 and 0.7 kb mRNA specify LHBs and HBx protein, respectively, 2.1 kb mRNA translate middle (MHBs) and small (SHBs) envelope proteins. The 3.5 kb mRNA contains pre-C mRNA and pgRNA. While the pre-C mRNA translates pre-core protein that is subsequently processed and secreted as e antigen (HBeAg), pgRNA translates core and polymerase proteins, in addition to serving as a template for reverse transcriptional DNA replication[4]. Similar to host cellular DNA, cccDNA in the nuclei of infected hepatocytes associate with nucleosomes to assemble into minichromosomes[33]. Transcription of cccDNA is regulated by ordered recruitment of specific chromatin modifiers and transcription factors as well as basal RNA polymerase II transcriptional complex.

Landscape of cccDNA-associated histone modification—Histone modifications play important roles in every aspect of chromatin functions, including DNA replication, transcription and repair as well as chromatin assembly and remodeling, by primarily serving as platforms for recruitment of other cellular proteins to participate and regulate chromatin metabolism. Using ChIP-Seq technology, cccDNA minichromosome genome-wide histone post-translational modifications (PTMs) had been examined. This study revealed that high levels of histone PTMs associated with active transcription, such as $H3K4^{me3}$, $H3K27^{ac}$ and $H3K122^{ac}$ are enriched at positions overlapping the four viral promoters[34]. Interestingly, IFN-α silence of cccDNA transcription is associated with the reduction of histone modifications specifying active transcription, but not the increase of histone modifications related to the silence of gene expression, such as $H3K9^{me3}$ and $H3K27^{me3}$ [34–35]. These findings suggest that while activation of cccDNA transcription is regulated by similar histone PTMs reported for cellular chromatin, silence of cccDNA transcription by IFN-α apparently works *via* a mechanism distinct from silencing of host chromatin transcription.

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As illustrated in Fig. 3, the active HBV cccDNA transcription state is attributed to the presence of HBx, a small viral regulatory protein[36]. In the absence of HBx, histone methyltransferase SETDB1 as well as histone deacetylases HDAC1 and Sirt1 are recruited to cccDNA minichromosomes, resulting in deposition of transcriptionally suppressive histone makers and erasing of active histone markers[37]. On the contrary, the presence of HBx can counteract SETDB1 mediated suppression, as well as bring in acetyltransferases p300/CBP, histone lysine demethylase-1 (LSD1) and other factors to establish transcriptionally permissive cccDNA state[37–38]. In addition to directly participating in cccDNA epigenetic coding, HBx was demonstrated to facilitate cccDNA transcription by counteracting the recruitment of a tudor domain protein Spindlin1 onto cccDNA which otherwise serves as a transcription suppressor[39], inhibiting protein arginine methyltransferase1 (PRMT1) activity to relieve PRMT1-mediated repression on cccDNA transcription[40], and stabilizing transcription factor p-CREB[41]. Most importantly, recent studies indicated that cccDNA minichromosome is transcriptionally silenced by the binding of cellular structural maintenance of chromosomes 5/6 (SMC5/6) complex, including Smc5, Smc6, Nse1, Nse2, Nse3, and Nse4 proteins[42]. Moreover, it appears that SMC5/6 complex suppression of cccDNA transcription requires its co-localization with nuclear domain 10 bodies (ND10), a nuclear protein aggregates containing PML, Sp100 and other proteins[42c]. By interacting with DNA-damage binding protein 1 (DDB1), HBx protein recruits DDB1-Cullin4 E3 ubiquitin ligase complex to degrade SMC5/6 complex and relieves its restriction on cccDNA transcription[42]. As a key regulator of viral infection, the stability of HBx is regulated by poly-ubiquitination mediated proteasomal degradation and E3 ligase HDM2 mediated Neddylation that stabilizes HBx[43].

Recently, a suppressive histone modification, symmetric dimethylation of H4R3, $H4R3^{me2s}$, catalyzed by protein arginine methyltransferase PRMT5 was found on cccDNA minichromosome even in the presence of HBx, leading to epigenetic silencing of cccDNA[44]. Interestingly, the recruitment of PRMT5 to cccDNA was dependent on HBV core protein, suggesting a role of core protein in regulating cccDNA transcription. Furthermore, binding of core protein to cccDNA CpG islands is associated with an epigenetic permissive state of cccDNA[45]. It is conceivable that selectively disrupting distinct interactions between HBx and/or core protein with their host binding partners might provide a chance to dismantle their roles in supporting cccDNA transcription and consequentially inactivate cccDNA, which may result in a functional cure of chronic hepatitis B[9].

In addition to histone modifications, cccDNA methylation also regulates its transcriptional activity. There are three CpG islands in HBV genome. Studies have shown that patients with high methylation frequency in CpG islands 2 and 3 are associated with lower viremia and serum HBV surface antigen (HBsAg) levels, respectively, probably due to the reduced transcription activity of HBV X and S genes[46]. In addition, hypermethylation in CpG islands 1 and 3 is associated with increased risk of HCC[47]. However, the cellular DNMT(s) that catalyze cccDNA CpG methylation remains to be identified.

Transcription factors and cccDNA transcription regulation—As illustrated in Fig. 2 and reviewed recently[48], the transcription activity of every HBV promoter or enhancer is regulated by multiple ubiquitous and hepatocyte enriched transcription factors. Particularly,

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the hepatocyte nuclear factors (HNF), including HNF1α, HNF3β, HNF4α, HNF6 and C/EBP, play the most important roles in cccDNA transcription and, at least in part, specify the hepatotropism of HBV[48a]. However, the regulatory activities of some transcriptional factors vary among the different experimental systems utilized. For instance, it has been demonstrated that HNF1α stimulates transcriptional activity of pre-S1 promoter and Enh II in HBV replicon plasmid-transfected hepatoma cells[49]. However, HBV transcription was not affected, whereas HBV DNA replication was modestly increased with appearance of cccDNA in HNF1α-null HBV transgenic mice[50]. Because the studies on HBV transcription have been done mostly in transiently transfected human hepatoma cells or HBV transgenic mice that cccDNA is not the transcription template, the biological function of those transcription factors in HBV cccDNA transcription should be further evaluated in HBV infected human hepatocytes.

HBV transcription is regulated by many cellular factors, such as cytokines and hormones, by modulating the function and abundance of transcription factors and epigenetic landscape of cccDNA minichromosome. For examples, interferon (IFN)-γ regulates HNF4 and C/EBP levels to affect HBV transcription and replication[51]. IL-6 controls HBV transcription by reducing the levels of HNF4α and HNF1α[52]. Interleukin-4 (IL-4) down-regulates C/EBPα to inhibit the activity of HBV core and preS2 promoter activity and pgRNA synthesis[53]. NF-ĸB represses the HBV gene expression through repression of Sp1 and HNF4-mediated transcriptional activation[54]. Hepatocystin down-regulates HNF4 through the Ras/MAPK pathway to impair HBV replication, and is important for determining the susceptibility of HBV to IFN-γ[55]. The activation of ERK1/2, ASPK/JNK or PI3K-AKT-mTOR pathway suppresses HBV transcription by negative regulation of HNF4α in both Huh7 and HepG2 cells[56][57]. Estrogen represses transcription of HBV genes by up-regulating ER-α, which interacts with and alters binding of HNF-4α to the HBV Enh I[58]. On the contrary, androgen increases the transcription of HBV through direct binding to the androgen-responsive element sites in viral Enh I[59]. This may explain a higher HBV titer in male carriers and an increased risk of HCC.

Stability and maintenance of cccDNA

The cccDNA minichromosomes are stable in non-dividing hepatocytes and possibly persist throughout the lifespan of host cells[60]. The high stability of cccDNA is further highlighted by the fact that CRISPR/Cas9 targeted cleavage of cccDNA rarely results in the loss of cccDNA, because the cleaved products are efficiently re-circularized by cellular NHEJ DNA repair pathway[61]. As illustrated in Fig. 4, while an important role of cytolytic T lymphocyte killing of infected hepatocytes is clearly evident in the resolution of acute HBV infection, the non-cytolytic cure of infected hepatocytes also occurs and is essential for the clearance of viral infection[62]. However, how the host antiviral immune response non-cytolytically eliminates cccDNA from infected hepatocytes remains controversial. Interestingly, Lucifera and Xia *et al* reported that treatment of HBV-infected primary human hepatocytes with IFN-α, IFN-γ, TNF-α or lymphotoxin-β receptor (LTβR) agonists non-cytolytically reduces the amount of cccDNA[63]. Mechanistically, it was shown that these inflammatory cytokines induce the expression of APOBEC3A and APOBEC3B, which are recruited to cccDNA *via* interaction with HBV core protein and deaminate cytosines in the negative strand of

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cccDNA. Deamination of cccDNA results in uracil-DNA glycosylase cleavage of uracils to form apurinic/apyrimidinic sites, which are subsequently recognized and cleaved by apurinic/apyrimidinic (AP) endonucleases[63a]. Interestingly, while a recent study demonstarted that while incubation of HBV replicating human hepatoma cells (Hep G2.1.15) with HBV-specific T-cell receptor mRNA-transfecetd resting human T lymphocytes non-cytolytically reduced HBV cccDNA by activation of APOBEC3 expression[64], IFN-α-induced reduction of cccDNA in HBV-infected HepaRG cells and/or PHHs was not obviously noted in other studies[65].

Another potential mechanism of cccDNA elimination is loss or dilution through cell division. Due to the lack of kinetochore structure, episomal DNAs are not equally partitioned into daughter cells during cell division and may be left in the cytoplasm and degraded after the cell division. Other DNA viruses, such as Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV) and bovine papillomavirus (BPV), tether their episomal genomes onto host chromosomes *via* interacting with a host bromodomain containing protein BRD4, to survive cell division[66]. A previous study in HBV infected chimeric mice suggested that both dilution of the cccDNA pool among daughter cells and significant loss of cccDNA occur during hepatocyte proliferation[67]. By tracking cccDNA fate with fluorescence *in situ* hybridization (FISH) technology, a recent study obtained evidence suggesting that cccDNA are symmetrically distributed into daughter cells[68]. However, there is no evidence suggesting that cccDNA are attached to metaphase chromosomes[68]. It is conceivable that therapeutic disruption of cccDNA distribution into daughter cells during hepatocyte proliferation should accelerate cccDNA decay and facilitate resolution of chronic HBV infection.

pgRNA splicing

Unlike classic retroviruses, RNA splicing is not required for HBV to produce viral envelope or regulatory proteins. However, pgRNA splicing products are readily detectable in the livers infected with HBV, woodchuck hepatitis virus (WHV) or DHBV[69]. The first HBV splicing product SP1RNA, a 2.2 kb RNA that shared the 5' cap and 3' poly(A) tail with pgRNA but with deletion of sequence between the last codon of core and a portion of Pol and S ORFs, was discovered in 1989 (Fig. 5, Example A). Up to now, more than 18 single or multiple spliced HBV RNA species have been identified. In cells actively transcribing HBV pgRNA, spliced (sp) RNA consists 10–30% of total pgRNA[70]. Splicing activity of pgRNA is regulated by the HBV post transcriptional regulatory element (PRE)[70b, 71] and host splicing related factors such as La and PSF[72].

Although Core and Pol ORFs are disrupted, many spRNA species have intact 5' epsilon (ε) structure and can thus be packaged into capsids and reverse transcribed into DNA. Because polymerase provided *in trans* seems in favor of *in situ* priming of positive strand DNA synthesis, the spRNA-derived DNA are largely dslDNAs, which are preferred substrates for integration into host chromosomal DNA[73]. In PLC/PRF/5 hepatoma cells, integrated HBV DNA had a deletion between splice donor (nt 2447) and acceptor (nt 2902) sites, indicating that the integrant is probably originated from spHBV DNA[74]. Moreover, levels of defective HBV particles derived from spRNA have been recently shown to be correlated with disease

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status. Particularly, presence of mutant HBV with preS1 deletion between aa 57 and 119, a consequence of alternative splicing, is significantly associated with development of chronic hepatitis, cirrhosis and HCC[75].

Although it is not absolutely required, the role for spRNA and their derived DNA or proteins in modulating HBV infection and pathogenesis cannot be ruled out. For instance, co-transfection of a spliced 2.2 kb RNA with wild-type HBV in HepG2 cells significantly enhanced HBV replication, in an HBx-dependent manner[76]. It was also reported that transfection of SP1 RNA into Huh7 cells up-regulated the expression of HBcAg and HBeAg from the cotransfected HBV replicon[77]. Interestingly, DHBV large surface protein can be translated from either preS1 promoter-directed authentic mRNA or a transcript derived from pgRNA splicing. Ironically, the presence of spliced preS1 mRNA was crucial for virion production in both animals and primary duck hepatocytes, but not in immortalized cell lines[69c].

Some of the spRNAs can support the synthesis of novel viral proteins which are collectively named as HBV splicing-generated proteins (HBSPs). To date, there are only two spliced RNAs that have been reported to be translated into HBSPs. The first one was found to form a 43 kD fusion protein that contained the N-terminal 47 aa of pol and the C-terminal 19 aa of preS1, plus the following preS2 and S domains (Fig. 5, Example A). This fusion protein lost the preS1 myristylation site but retained the core interacting domain and surface protein transmembrane domains. The fusion protein was incorporated into both Dane and subviral particles[78]. Another HBSP is a 10 kD fusion protein derived from SP1 RNA. It contains 46 amino acid (aa) residues from the N terminal of Pol and 65 aa residues from a frame shift after nt 489 (Fig. 5, Example B)[79]. In the 1.3.32 HBV transgenic mice, treatment with carbon tetrachloride (CCL4) or lipopolysaccharide (LPS) induced a significant increase of SP1 RNA in the liver. The elevation of SP1 RNA inhibited the recruitment of monocytes and macrophages and subsequently down-modulated liver damage[72].

In summary, pgRNA splicing is regulated by host pathobiological factors. The advent of biologically relevant HBV infection cell culture and animal models should facilitate the investigation toward uncovering the roles of spRNA as well as derived proteins and DNA products in HBV infection and pathogenesis.

Viral RNA nuclear export and decay

Similar to retroviruses, to avoid aberrant RNA splicing, nuclear export of intron-less HBV RNA is facilitated by host cellular factors through interaction with post-transcriptional regulatory element (PRE) at the 3′ region of HBV transcripts. HBV PRE contains approximately 450 nucleotides spanning nt 1151 to 1582 and is divided into 3 sub-elements HPREα (nt 1151–1346), HPREβ1 (nt1347–1457) and HPREβ2 (nt 1458–1582)[80]. Unlike HIV PRE that interaction with viral protein Rev is required for full-length viral RNA nuclear export, HBV PRE mediated viral RNA nuclear export does not require the involvement of any viral protein. Instead, cellular lupus-associated (La) protein and polypyrimidine tract binding protein (PTB1) have been found to bind the HPREα and HPREβ2 region and consequentially stabilize and facilitate HBV mRNA nuclear export, respectively[81]. Intriguingly, it was reported recently that a small molecule dihydroquinolizinone, 6-R2-10-

methoxy-9-R1-2-oxo-2,3,4,6,7,11b-hexahydro-1H-pyrido[2,1-a]-isoquinoline-3-carboxylicacid (DHQ-1), selectively induced HBV RNA degradation in a manner depending on a 109-nucleotide RNA element in HPREα region[82]. It was further demonstrated that DHQ-1 specifically interacts with host cellular proteins non-canonical poly(A) polymerases D5 (PAPD5) and D7 (PAPD7), and knocking down the expression of these cellular proteins reduces HBV RNA and HBsAg expression[83]. It will be extremely interesting to know how the cellular 3'-end polymerases stabilize HBV RNA and how their interaction with DHQ-1 results in HPREα sequence-dependent HBV RNA degradation.

HBV mRNAs are also targeted by host innate and adaptive immune response to control viral infection. For example, adoptive transfer of HBV-specific CTLs into HBV transgenic mice induces HBV RNA decay, which is tightly associated with the cytokine-induced proteolytic cleavage of La, a protein that binds HPREα element and stabilize HBV RNA[81a, 84]. In addition, investigation of IFN antiviral mechanism against HBV reveals two IFN-induced cellular proteins, zinc finger antiviral protein (ZAP) and ISG20, that selectively degrade HBV RNA through interaction with HBV RNA terminal redundant region containing ε stem-loop structure[85].

pgRNA packaging and reverse transcription

HBV DNA synthesis occurs in cytoplasmic nucleocapsids by viral DNA polymerase self-primed reverse transcription of pgRNA. The ε signal located on the 5' end of pgRNA is recognized by the viral polymerase to initiate pgRNA packaging. The roles of HBV core protein and polymerase as well as related host factors in pgRNA packaging and reverse transcription have been extensively investigated[86].

Cellular chaperone proteins play critical role in pgRNA packaging—Early studies indicate that the stem-loop structure ε located at the 5' end of pgRNA is its packaging signal and viral DNA polymerase is required for pgRNA package by core protein[86]. It was soon discovered that Hsp90 is required for viral polymerase to bind ε RNA element, an essential step for the packaging of viral polymerase-pgRNA complex into nucleocapsids and the priming of negative strand DNA synthesis[87]. *In vitro* reconstitution studies elegantly demonstrated that Hsp90 and its co-chaperones, Hsp70, Hsp40, Hop/p60, and p23, are all required for viral polymerase binding of pgRNA and priming of DNA synthesis[88]. In addition, through mediating interaction between viral polymerase and Hsp90, co-chaperone p50 increases pgRNA packaging and DNA replication[89]. While p23 has been shown to be packaged with viral polymerase and pgRNA into nucleocapsids, whether other chaperones are co-assembled has not been experimentally determined[90].

Other host factors regulate pgRNA encapsidation—In addition to chaperones, protein arginine methyltransferase 5 (PRMT5) has been reported to inhibit pgRNA encapsidation. One study showed that PRMT5 binds to the RNase H domain of viral DNA polymerase and prevents its interaction with pgRNA, which results in inhibition of pgRNA packaging and DNA replication in a methyltransferase activity-independent manner[44]. Another study showed that PRMT5 interacts with the HBV core protein and di-methylates arginine residues R150 and R156 in the CTD of core protein to modulate its subcellular

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distribution. While symmetric di-methylation favors core protein nuclear accumulation, monomethylation of core protein results in cytoplasmic accumulation[91]. The altered intracellular trafficking of core protein should not only interfere with capsid assembly, but also affect core protein-mediated PRMT5 recruitment and the formation of H4R3^{me2s} repressive mark on cccDNA minichromosome[44]. Moreover, it has been known for a long time that IFN-α treatment inhibited pgRNA encapsidation[92], but the detailed mechanism involved in the cytokine-induced cellular response leading to disruption of pgRNA packaging remains elusive.

Core protein dynamic phosphorylation regulates pgRNA packaging—HBV core protein consists of N-terminal assembly domain and arginine-rich CTD with seven conserved serines or threonine that are dynamically phosphorylated/dephosphorylated during the viral replication cycle (Fig. 6). Phosphomimetic mutagenesis and metabolic labeling studies indicated that CTD phosphorylation, particularly at S162 and S170, is required for pgRNA packaging. Our recent studies showed that pgRNA encapsidation is associated with core protein dephosphorylation[93]. Interestingly, while studies with HBV and DHBV demonstrated that CDK2 is assembled into nucleocapsids and phosphorylates core protein[94], several other cellular kinases, such as SRPK1, SRPK2[95], PKA, CK2-aPKAIα/CK2-aPKAIIα, GAPD-PK, PKC, and PLK1, have also been reported to phosphorylate one or multiple phosphoaceptor sites in core protein CTD[96]. As depicted in Fig. 6, it has been reported that S155, S162 and S170 can be phosphorylated by CDK2[94, 97], S168, S176, and S178 can be phosphorylated by PLK1 and PKA, S155, S162, S168, S170, S176, S178 and S181 can be phosphorylated by SRPK[98]. Interestingly, it appeared that phosphorylation of S168, S176 and S178 is dependent on prior phosphorylation of S155, S162 and S170[99]. Cellular phosphatases that dephosphorylate core protein have not been identified.

Host functions regulate reverse transcription—HBV DNA synthesis in nucleocapsids is also regulated by host cellular factors. For example, although only 5 to 10% of DHBV genome in DHBV replicating cells are dslDNA, *in vitro* synthesis of DHBV DNA from purified pgRNA-containing capsids yields predominantly dslDNA[22a], suggesting that the primer translocation/genome circularization is facilitated by host cellular factor(s) that are lost during the purification of pgRNA-containing capsids. In agreement with this observation, a recent study showed that the increased proportion of dslDNA in circulating HBV virions is associated with liver inflammation[100]. Furthermore, antiviral protein APOBEC3G can be packaged in nucleocapsids to inhibit reverse transcription and deaminate viral negative strand DNA, the later activity results in G to A hypermutation of HBV DNA[101]. However, although translation initiation factor eIF4E was demonstrated to be encapsidated with viral polymerase and pgRNA, its biological function has not been determined[102].

Cellular functions are required for secretion of viral and subviral particles

As illustrated in Fig. 1, in addition to double-stranded DNA-containing mature nucleocapsids, viral RNA-containing capsids and empty capsids can also be enveloped and secreted out as virion-like particles, or RNA-containing virions and genome-free (GF) virions, respectively[103]. Interestingly, core proteins are hyperphosphorylated in GF-virions,

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but dephosphorylated in DNA-containing virions, or complete virions. However, the dephosphorylation is not required for mature nucleocapsid envelopementation[104]. In the blood of HBV-infected individuals, GF-virions are usually more abundant than that of complete virions, but RNA-containing virions are less abundant than that of complete virions[105]. The RNA species in RNA-containing virions can be either pgRNA or spliced viral RNA[106]. How those different cytoplasmic capsids are selectively enveloped and secreted as virions or virion-like particles is poorly understood[103]. Accumulating evidence suggests that assembly and secretion of complete HBV virions depend on intraluminal vesicles of maturing endosomes, *i.e.*, the multivesicular bodies (MVB), and endosomal sorting complex required for transport (ESCRT) systems. Specifically, mature nucleocapsids are recognized by Nedd4. Ubiquitinated Nedd4 interacts with the ubiquitin-interacting adaptor γ2-adaptin and bridges membrane studded with envelope proteins and nucleocapsids to ESCRT machinery for virion assembly[107]. However, a recent study suggested that α-taxilin interacts with LHBs and the ESCRT I component tsg101 to recruit mature nucleocapsids to ESCRT machinery[108]. Nevertheless, the requirement of HBV morphogenesis on Vps4A/B and Alix/AIP1 implies that the pre-assembly complex utilizes the scission function of ESCRT-III/VPS4 to bud into the late endosomes or MVBs and exits cells by the exosome pathway[109].

Besides the virions and virion-like particles, HBV infected hepatocytes also secrete two types of subviral particles (SVPs) that contain only viral envelope proteins, but not capsids, the small spheres and filaments of 20 nm in diameter. SVPs exist in the blood of infected individuals at 1,000 to 100,000-fold in excess over virions. While the small spheres, that contain SHBs and MHBs, bud into the lumen of the ER/ERGIC and exit cells *via* the constitutive secretion pathway, the filements, that share similar viral envelope protein contents with virions[110], assembled and released *via* the ESCRT/MVB pathway like the infectious virions[111].

2. Cellular non-coding and viral RNAs regulate HBV replication and pathogenesis

Virus-host interactions can also be mediated by RNA molecules encoded by viral and cellular genomes, such as non-coding (nc) RNA and microRNA. Many cellular microRNAs have been reported to modulate HBV replication by either directly targeting viral RNA or indirectly targeting cellular mRNA encoding proteins regulating HBV infection, which have been reviewed recently[112]. Herein, we only discuss the regulatory effects of recently identified non-coding RNA transcribed from cccDNA or integrated HBV DNA on viral replication and pathogenesis.

Using cap analysis of gene expression technology to quantitatively map the global transcriptional start site (TSS) distribution over the entire HBV genome in liver tissues, Altinel and colleagues identified 17 robust TSSs, including all the classically identified transcripts and some minor transcripts. Importantly, two minor viral transcripts in antisense polarity were identified, which might be involved in the regulation of the neighboring promoter activity[113]. Moreover, Yang and colleagues identified a HBV encoded microRNA,

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designated as HBV-miR-3, with its sequence mapped to the highly conserved HBsAg coding region of various HBV genotypes. HBV-miR-3 can be derived from 2.1kb, 2.4kb or 3.5kb mRNA, and targets the 3.5 kb mRNA at the ORF region of core gene to specifically reduce core protein expression, levels of pgRNA, and consequential viral replication[114]. The biological significance of HBV-miR-3 in HBV infection and pathogenesis in humans remains to be determined.

As discussed in detail below, integrated HBV DNA can transcribe viral-human chimeric RNAs. For instance, integration of HBV DNA into a long interspersed nuclear elements (LINE) in a normally silent intergenic region, chr.8p11.21, results in the production of an oncogenic HBx-LINE1 chimeric transcript. HBx-LINE1 can be detected in 23.3% of HBV-associated HCC tumors and correlates with poorer patient survival. Functioning as a long non-coding RNA, HBx-LINE1 activates Wnt/β-Catenin signaling and promotes cell motility through epithelial-to-mesenchymal transition, which contributes to the ultimate development of liver cancer[115]. Interestingly, HBx-LINE1 RNA has six binding sites for miR-122, a major microRNA in hepatocytes with important functions in cellular metabolism, development and differentiation[116]. It is thus possible that the epithelial-to-mesenchymal transition promoted by HBx-LINE1 works through a miR-122-sequestering mechanism[117].

3. Genomic response of HBV infection and its biological consequences

Despite not a requirement for replication, HBV DNA occasionally integrates into chromosomal DNA of infected hepatocytes. Integration can be detected as early as a few days post infection and the frequency of integration increases in the infected livers with the duration of viral infection[118]. The dslDNA, a minor species of HBV genomic DNA derived from *in situ* priming of positive-stranded DNA synthesis, is the preferential substrate for integration[119]. Results obtained from genetic studies and sequence analyses of viral-host DNA junctions support the hypothesis that hepadnaviral DNA integration occurred at sites of host cell DNA damage, preferentially double strand DNA break, through non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) DNA repair pathway[120]. Not surprisingly, it is well documented that DNA damage promotes viral DNA integration[121]. Moreover, a recent study revealed that the abundance of dslDNA in the circulating virions increases under the condition of liver inflammation[100]. Hence, the increased levels of dslDNA and host cellular DNA damage in inflammatory livers may facilitate HBV DNA integration and promote oncogenesis of infected hepatocytes *via* activation of oncogenes, inactivation of tumor suppressor genes, inducing genome instability and other mechanisms. As illustrated in Fig. 7, due to the disruption and loss of HBV DNA sequence corresponding to the 5’ and 3’ terminus of dslDNA, the integrated HBV DNAs are not possible to produce an authentic pgRNA to support viral DNA replication. However, at least some of transcripts derived from integrated DNA contain complete open reading frame of viral envelope proteins and can thus translate HBsAg. In fact, the significant contribution of integrated HBV DNA to circulating HBsAg in humans, particularly in patients who are HBeAg-negative or receive long-term therapy with NUCs, is only appreciated recently in a clinical trial of HBV mRNA-targeting small interference RNA (siRNA) therapy[122]. In addition to full-length dslDNA, as mentioned above, dslDNA derived from spliced pgRNA can also integrated into host cellular chromosomes to produce viral RNA as well as

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truncated viral proteins, viral-viral and/or viral-host fusion proteins and RNAs to modulate host cellular functions and play important role in HBV carcinogenesis[123].

4. Virological basis for the cure of chronic HBV infection

Complete (or sterilized) cure of HBV infection means the eradication of all free viruses as well as killing and/or cure of all HBV-infected cells from an infected individual. Due to the fast clearance of cell-free viruses from circulation[124], cure of a HBV-infected hepatocyte or individual can be achieved by complete inhibition of HBV DNA replication for a period of time (T_{cure}) that allows the complete decay of the most stable HBV DNA replication intermediate, cccDNA. Obviously, the time required to cure an infected individual is determined by the fraction of infected cells with longest T_{cure}, such as the cells with very low rate of turnover and long life span. Selective killing of this fraction of HBV infected cells will accelerate the cure. Because complete inhibition of HBV DNA replication has not been achieved by current available antiviral agents[125], the T_{cure} for an infected individual is difficult to estimate. It is our hope that combination therapy of multiple HBV DNA replication inhibitors, such as NUCs and CpAMs, will more efficiently inhibit HBV replication and cure the HBV infection with a finite, ideally a reasonably short T_{cure}. However, accumulating evidence suggests that immunological resolution of acute HBV infection is not a complete cure, but a tight immune control of a very low level of residual HBV infection (replication)[126]. Therefore, the complete cure of HBV infection is desirable, but may not be realistic. Practically, the therapeutic goal of chronic hepatitis B is to achieve a sustained loss of HBsAg or more ideally, anti-HBs seroconversion, an indication of stable immune control of HBV infection or a functional cure[127]. In addition to complete inhibition of viral replication by combination antiviral therapy[24a] and selective killing of HBV-infected cells by immunotherapies[128], elimination or transcriptional silencing of pre-existing cccDNA in the nuclei of infected hepatocytes will cure HBV infected cells or reduce viral antigen load, which may be essential for restoration of functional host antiviral adaptive immune response and ultimately achieve a functional cure[5, 9]. Although it has been demonstrated that CRISPR/Cas9 or other DNA editing technologies can efficiently mutate and functionally inactivate cccDNA in HBV infected hepatocytes[61], it is a challenge to deliver the DNA editing molecules into every HBV-infected hepatocytes to inactivate all the cccDNA molecules.

To therapeutically silence cccDNA transcription, it is essential to specifically target the transcription factors or epigenetic modifications that are unique to cccDNA minichromosomes. Unfortunately, such unique cccDNA transcription regulation properties have not yet been identified. However, recent findings about the critical role of HBx protein in cccDNA transcription regulation suggest that HBx itself is an ideal target for the future discovery of small molecules towards permanent silencing of cccDNA transcription. For instance, small molecules that selectively bind HBx to disrupt its interaction with Cullin4-DDB1-E3 ligase complex would inhibit degradation of Smc5/6 in infected hepatocytes and restore the heterochromatin status of cccDNA minichromosomes (Fig. 3). As discussed in previous sections, several lines of circumstantial evidence indicate that HBV core protein may also bind cccDNA minichromosome and regulate its transcriptional activity. If this is

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the case, disruption of core protein interaction with cccDNA is also an ideal therapeutic target to silence cccDNA function.

As a stealthy virus, HBV escapes cell intrinsic antiviral mechanisms through avoiding recognition by pattern recognition receptors (PRRs) rather than blocking its effecter functions. In line with this notion, HBV replication can be inhibited by agonists of several endosomal and cytoplasmic PRRs[129]. Due to the difficulty to eliminate residual amounts of HBV cccDNA from infected individuals, a functional cure requires activation of host adaptive antiviral immune response, particularly, HBV-specific cytolytic T cell response, to maintain a sustained surveillance of HBV reactivation.

Finally, recent findings that integrated HBV DNA serves as the significant contributor of HBsAg in circulation as well as HBx-LINE1 RNA plays an important role in HCC oncogenesis highlight the importance to therapeutically target integrated DNA and their transcripts for the functional cure of chronic HBV infection and prevention of HCC development. While CRISPR/Cas9-targeted cleavage and inactivation of integrated DNA may result in chromatin DNA break, RNA interference and small molecules specifically inhibiting cellular PAPD5/7 have been demonstrated to selectively induce degradation of viral RNA transcribed from cccDNA and integrated HBV DNA. It is anticipated that further investigation of HBV-host interaction may reveal novel molecular targets for safer and more efficient elimination or inactivation of cccDNA, restore host antiviral immune response and ultimately cure chronic HBV infection.

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Conflict interest statement

The authors declared the following competing financial interest(s): J.-T. G receives research fund from and holds stock of Arbutus Biopharma, Inc. J.C. receives research fund from Arbutus Biopharma, Inc..

BRBREVIATIONS

cccDNA	covalently closed circular DNA
CpAM	core protein allosteric modulator
DDB1	DNA-damage binding protein 1
DHBV	duck hepatitis B virus
DHQ-1	dihydroquinolizinone
dslDNA	double-stranded linear DNA
EBV	Epstein-Barr virus
ESCRT	endosomal sorting complex required for transport

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HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HNF	hepatocyte nuclear factor
HSPG	heparan sulfate proteoglycan
ISG20	interferon stimulated gene 20
KSHV	Kaposi's sarcoma-associated herpesvirus
MMEJ	microhomology-mediated end joining
MVB	multivesicular bodies
NHEJ	non-homologous end joining
NLS	nuclear localization signal
NPCs	nuclear pore complexes
NTCP	sodium taurocholate cotransporting polypeptide
PAPD5	poly(A) polymerases D5
pgRNA	pregenomic RNA
PRE	post-transcriptional regulatory element
PRMT	protein arginine methyltransferase
rcDNA	relaxed circular DNA
SMC5/6	structural maintenance of chromosomes 5/6
TDP2	Tyrosol-DNA phosphodiesterase 2
WHV	woodchuck hepatitis virus
ZAP	zinc finger antiviral protein

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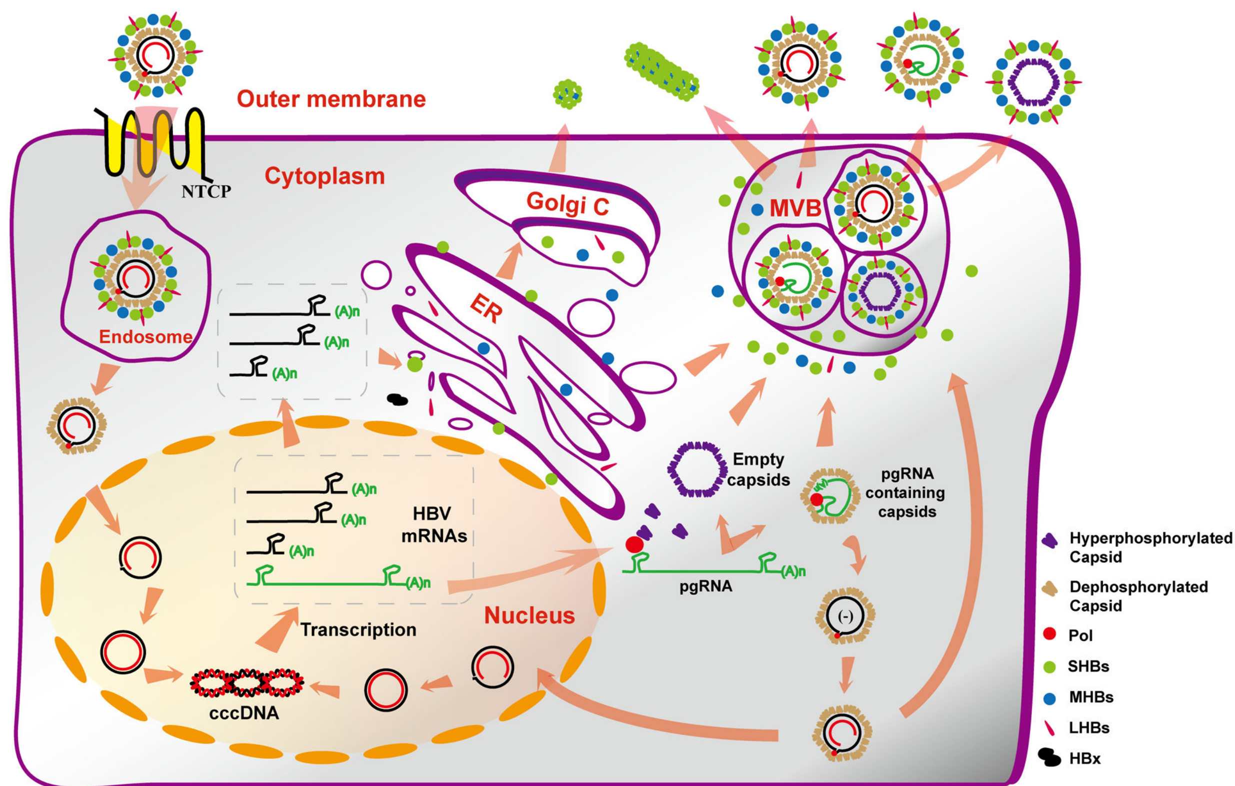

Figure 1. Schematic representation of HBV replication cycle.

HBV binds to its cellular receptor NTCP to trigger endocytosis that internalizes virion into endosome where the nucleocapsid is released into the cytoplasm. The viral genomic DNA in the nucleocapsid is transported into the nucleus and converted into cccDNA, which serves as the template to transcribe viral RNAs. Viral DNA polymerase binds to pgRNA and is subsequently assembled by core proteins to form nucleocapsids where the pgRNA is reverse transcribed into DNA. Core protein also assembles into empty capsids. Both viral DNA- (to a lesser extent, RNA-) containing or empty capsids can be enveloped and secreted as virions or empty virions. Meanwhile, viral envelope proteins also assemble and secrete large amounts of small sphere and filament subviral particles. See text for more detailed explanation.

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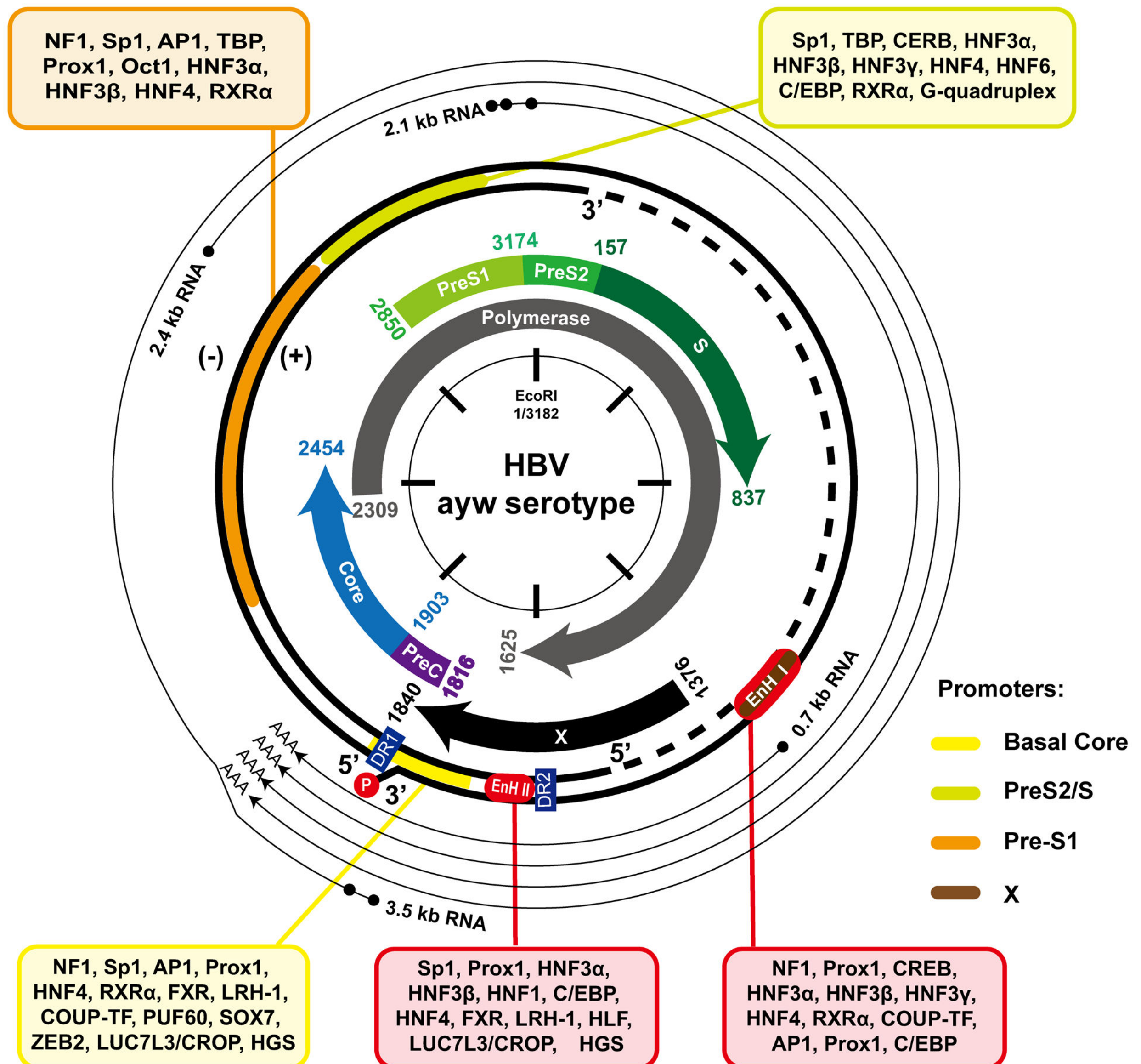

Figure 2. HBV genome structure and transcription.

The relaxed circular (rc) DNA genome structure, ORFs and cis-transcriptional regulation elements are depicted. The transcription factors bound to each of the transcriptional elements are presented. The nucleotide numbering is based on genotype D HBV (ayw).

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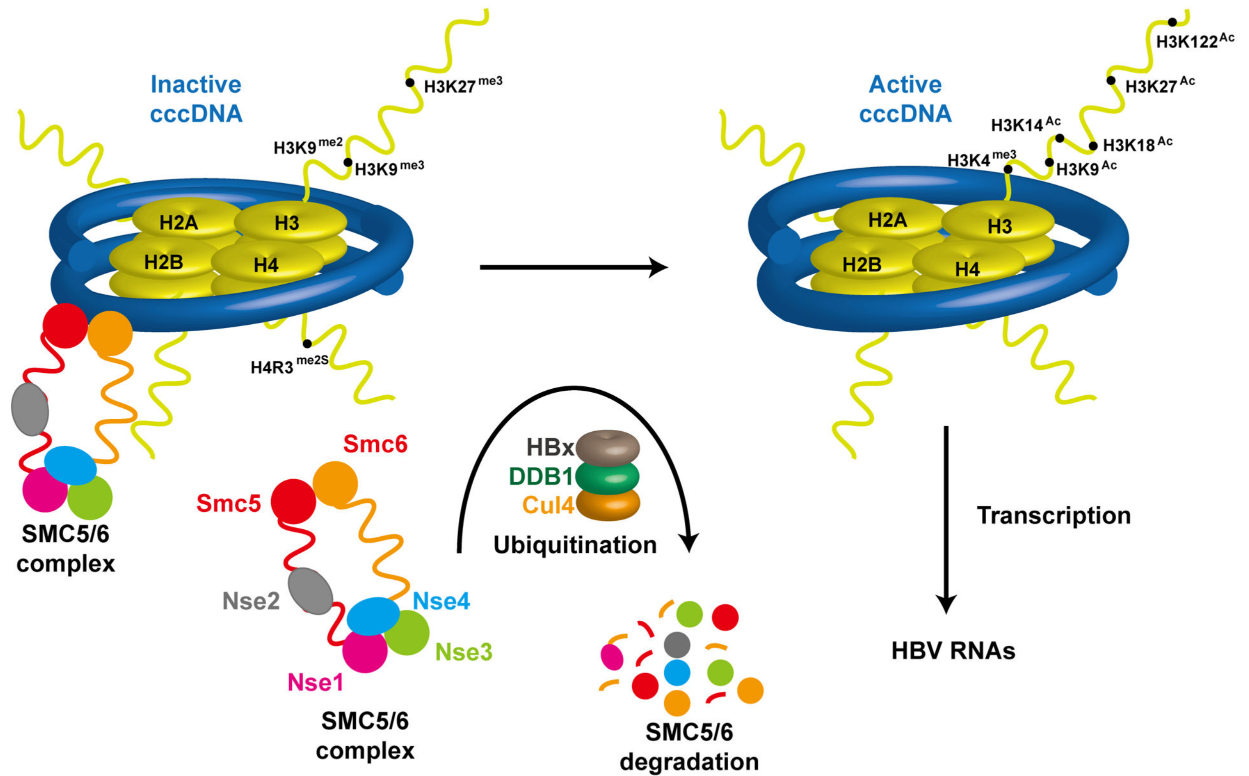

Figure 3. Epigenetic regulation of HBV cccDNA transcription by HBx protein.

Binding of SMC5/6 complex to cccDNA maintains the cccDNA in a transcriptionally silencing state, characterized by H3K9^{me3}, H3K29^{me3} and H4R3^{me2s}. Through interaction with DDB1, HBx recruits E3 ubquitin ligase Cullin 4 to degrade SMC5/6 complex and induces the establishment of a transcriptionally permissive cccDNA state.

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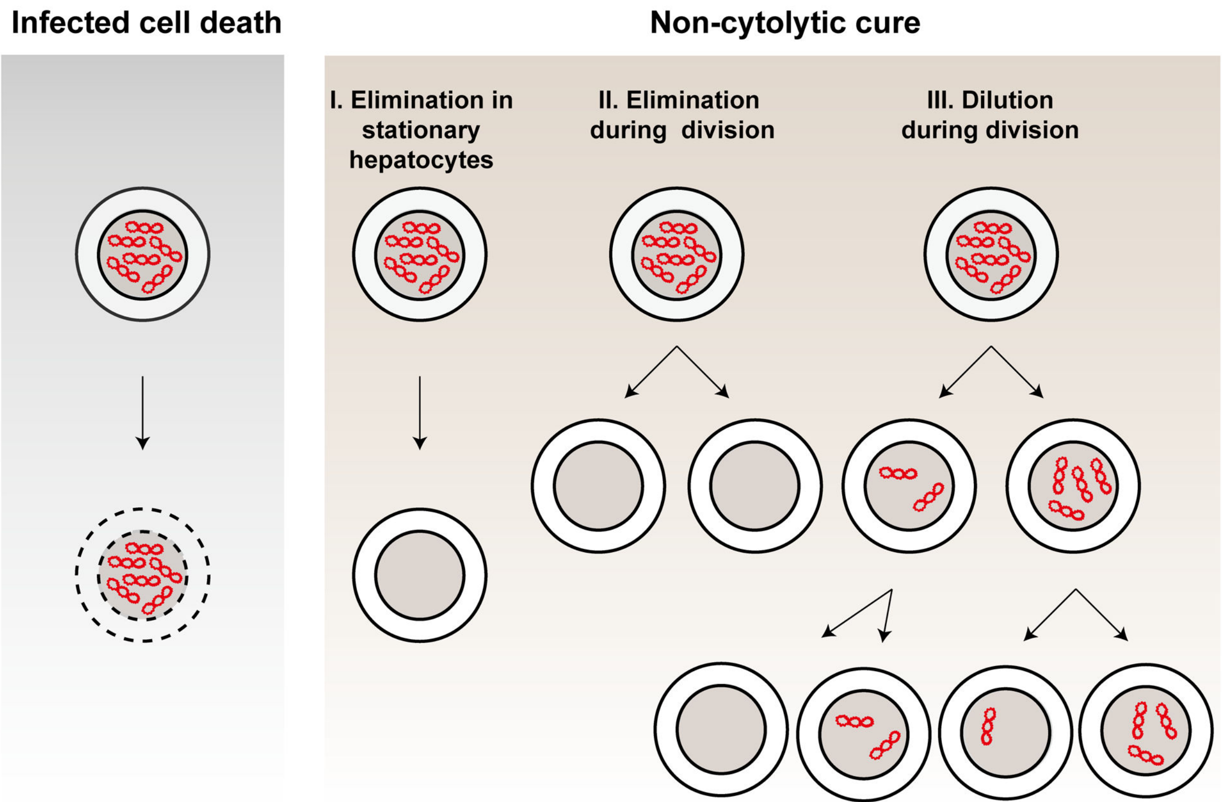

Figure 4. Mechanisms to eliminate cccDNA.

Pre-existing cccDNA can be eliminated by killing of infected hepatocytes (A) or non-cytolytically cure of infected hepatocytes *via* three distinct mechanisms (B).

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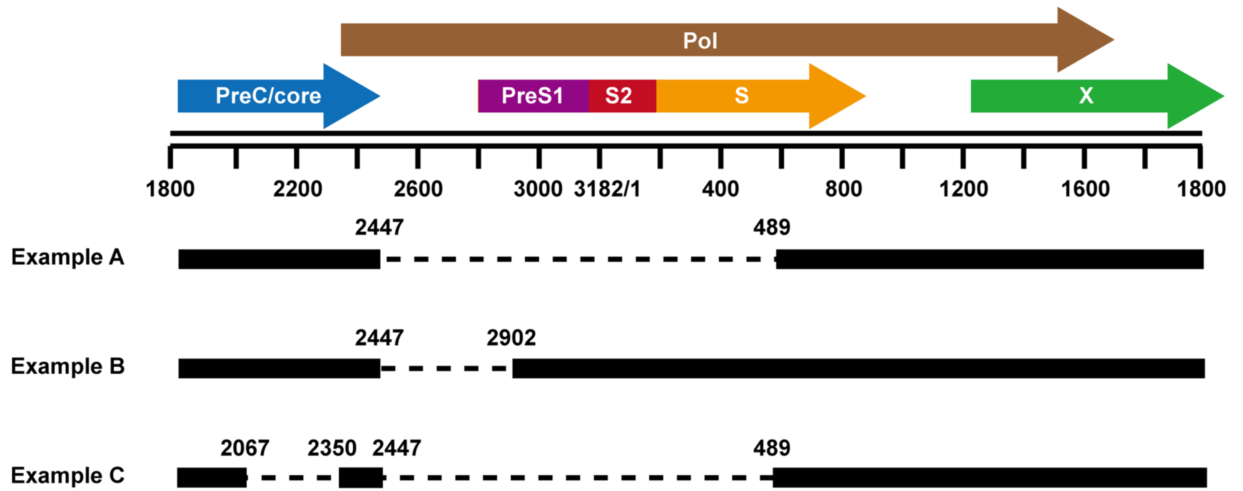

Figure 5. Examples of pgRNA splicing.

In pgRNA, the nucleotides 2447 and 2007 are two most common splicing donor sites. The nucleotides 489, 2902 and 2355 are common splicing acceptor sites. Three examples of single and double spliced pgRNA are depicted. Examples A and B produce fusion proteins that modulate virion morphogenesis and cell death pathway, respectively.

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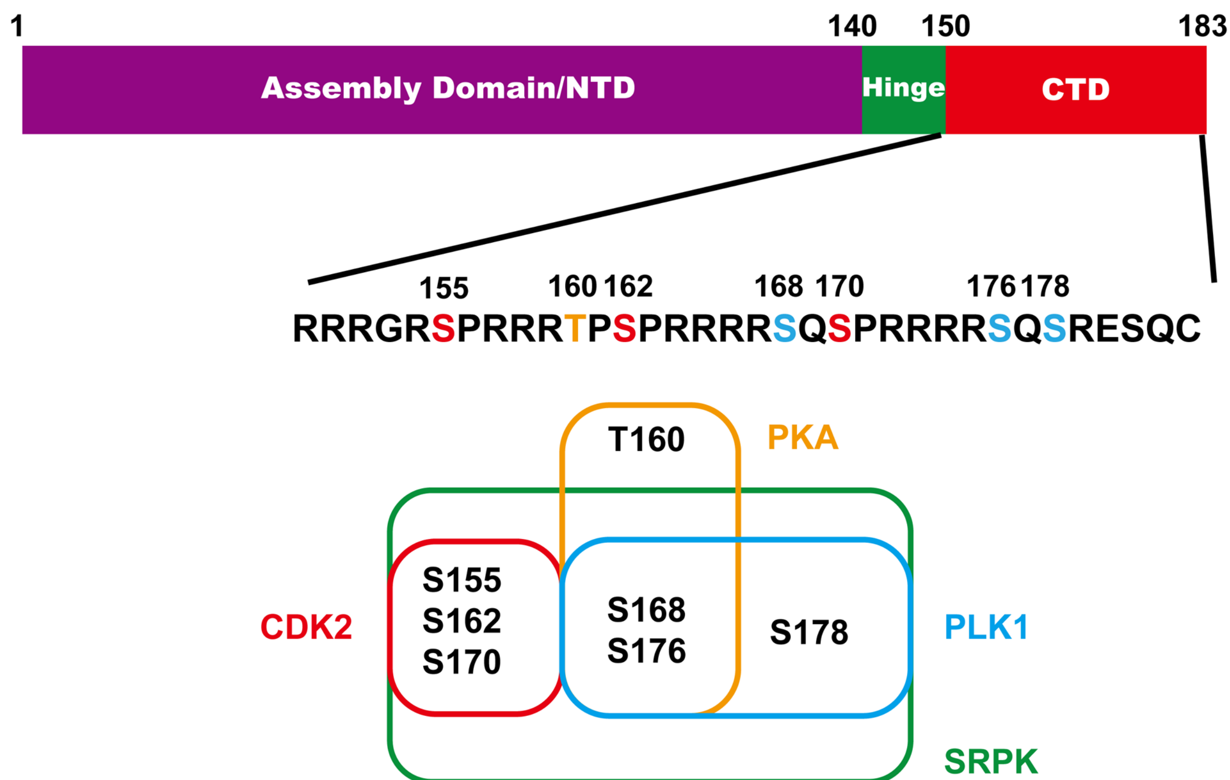

Figure 6. HBV core protein and CTD phosphorylation.

The domain structure of core protein and sequence of CTD domain are presented. The three major (red) and four minor (blue) phosphoacceptor sites are highlighted. Kinases and their ability to phosphorylate the specific serines/thorenine are also depicted.

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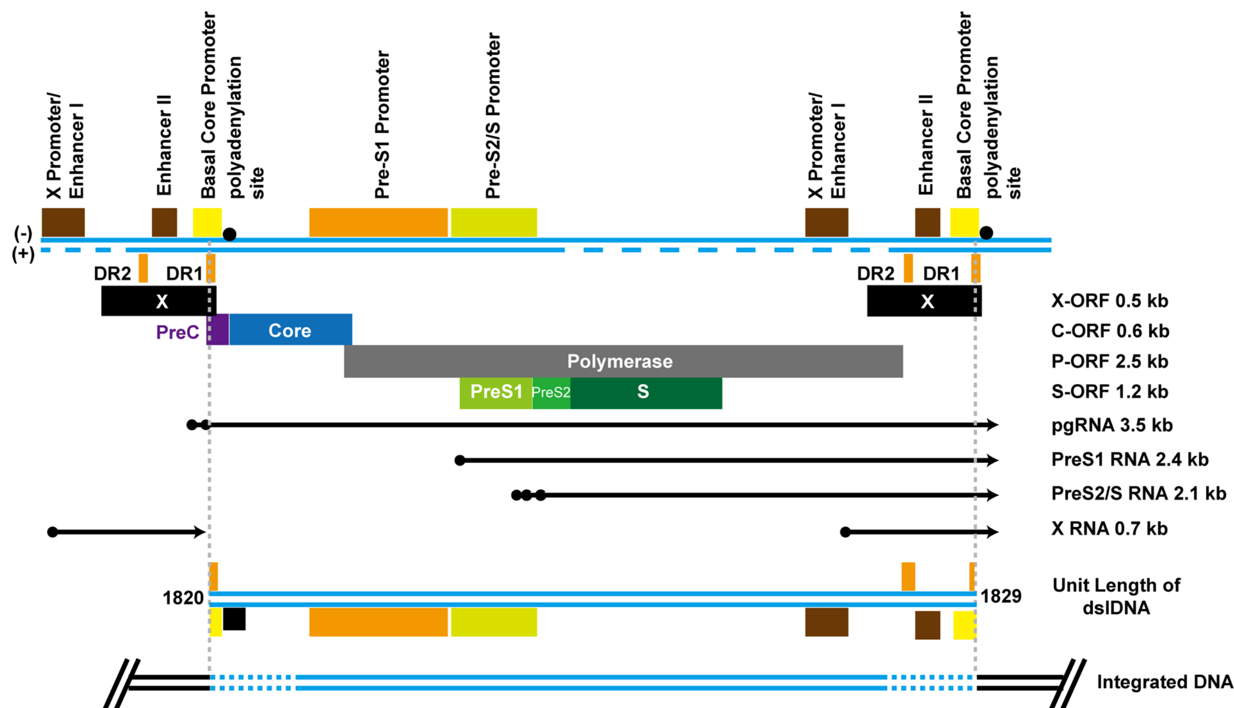

Figure 7. Structure and function of integrated HBV DNA.

Top panel, HBV transcriptional *cis* regulatory elements, ORFs, and transcripts are presented in reference to linear DNA longer than unit-length of HBV genome. *Middle panel*, the structure of HBV DNA double stranded linear (dsl) DNA genome is presented. *Low panel*, the structure of integrated HBV DNA is presented. Due to the processing of the dslDNA ends during integration, variable length of DNA sequences may be lost (dashed lines).