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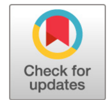
MINIREVIEW



Revisiting Hepatitis B Virus: Challenges of Curative Therapies

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ABSTRACT With a yearly death toll of 880,000, hepatitis B virus (HBV) remains a major health problem worldwide, despite an effective prophylactic vaccine and well-tolerated, effective antivirals. HBV causes chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. The viral genome persists in infected hepatocytes even after long-term antiviral therapy, and its integration, though no longer able to support viral replication, destabilizes the host genome. HBV is a DNA virus that utilizes a virus-encoded reverse transcriptase to convert an RNA intermediate, termed pregenomic RNA, into the relaxed circular DNA genome, which is subsequently converted into a covalently closed circular DNA (cccDNA) in the host cell nucleus. cccDNA is maintained in the nucleus of the infected hepatocyte as a stable minichromosome and functions as the viral transcriptional template for the production of all viral gene products, and thus, it is the molecular basis of HBV persistence. The nuclear cccDNA pool can be replenished through recycling of newly synthesized, DNA-containing HBV capsids. Licensed antivirals target the HBV reverse transcriptase activity but fail to eliminate cccDNA, which would be required to cure HBV infection. Elimination of HBV cccDNA is so far only achieved by antiviral immune responses. Thus, this review will focus on possible curative strategies aimed at eliminating or crippling the viral cccDNA. Newer insights into the HBV life cycle and host immune response provide novel, potentially curative therapeutic opportunities and targets.

KEYWORDS *Hepadnaviridae*, hepatitis B virus, hepatocellular carcinoma, interferons, reverse transcriptase, cccDNA

A diverse group of human viruses that primarily infect hepatocytes constitute the human hepatitis viruses. Of all the human hepatitis viruses, only hepatitis B virus (HBV) and hepatitis C virus (HCV) cause acute and chronic infection and have similar pathologies and clinical outcomes. Both viruses cause chronic inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma, but HBV and HCV are two entirely different viruses. While both viruses are transmitted by direct blood contact, only HBV is transmitted vertically from mother to child (1). HBV infection during early childhood has a >90% risk of leading to chronic hepatitis and accounts for the highest number of chronic carriers, despite the availability of an effective prophylactic vaccine. HBV belongs to the *Hepadnaviridae* family, a group of pararetroviruses that replicate via reverse transcription (2, 3) and express viral proteins from their nuclear transcription template, the covalently closed circular DNA (cccDNA). HCV, in contrast, is a positive-strand RNA virus belonging to the *Flaviviridae* family that does not have a persistent genomic form. It therefore needs to replicate constantly to persist, allowing efficient targeting by antivirals. In addition, the HCV positive-polarity genome is translated into a polyprotein that requires extensive processing to produce the final viral protein products and thereby offers a number of targets for antiviral drugs (4–6). While there

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is no hepatitis C vaccine in sight, new direct-acting antivirals (DAA) are phenomenally effective, with more than 98% efficacy in curing viral infection (7). For HBV, so far, only the nucleos(t)ide analogues (NUC) are available as DAA, but they are poorly effective in curing persistent infection. The remarkable success in curing HCV infection has fueled the interest of basic scientists and the pharmaceutical industry alike in turning or returning to HBV with the aim of finding curative therapeutic schemes.

Initially identified as the Australian antigen by Baruch Blumberg, which won him the Nobel Prize, HBV was discovered in 1965 (8), and in the following years, it was established that it was a DNA virus with an endogenous DNA polymerase activity (9, 10). In 1979, four groups reported the molecular cloning of the HBV DNA genome from infected patients (11–14). In 1982, Summers and Mason found that HBV polymerase reverse transcribes an RNA intermediate into an incomplete and relaxed circular DNA (rcDNA), which later is transformed into the cccDNA in the host cell nucleus and forms the template for viral transcription (15). These seminal discoveries placed HBV on an experimental platform and, thus, launched an era of HBV molecular biology that led to the development of the recombinant HBV vaccine consisting of just the small (S) HBV envelope protein assembling to form the HBV surface antigen (HBsAg) (16, 17). It represents the first vaccine ever based on recombinant DNA technology.

Although HBV can be cleared in adults following a transient, acute infection, which is estimated to have afflicted some 2 billion people worldwide, the virus persists and causes chronic infection, especially when transmitted neonatally or to a toddler (18, 19). Persistent HBV infection is one of the leading risk factors for the development of hepatocellular carcinoma (HCC) (20). Compelling epidemiological evidence shows that about 54% of all HCC is associated with HBV infection. HBV is a noncytopathic virus and does not encode a prototype oncogene. Liver damage is caused by the host immune response that results in inflammation, apoptosis, and regeneration, leading to the accumulation of genetic and epigenetic changes. Genomic instability due to HBV DNA integration and impaired DNA repair may also be a significant contributing factor in the hepatocarcinogenesis. In addition, the indirect oncogenic role of the viral regulatory protein HBx (see below) in the process has been the subject of intense debate. A large body of data has attributed various biological activities to HBx, including alteration of epigenetics and signal transduction pathways, induction of oxidative stress, effects on DNA repair, alteration of chromosomal dynamics, and effects on cellular homeostasis (21, 22). In sum, the most prevalent view of HBV's oncogenic role in HCC development is that the viral infection, which spans decades, induces continuous liver inflammation and regeneration of hepatocytes and, in the process, the integration events and mutations that accumulate can activate oncogenic induction with the derailed DNA repair process.

Despite the highly effective (prophylactic) vaccine, HBV infection remains a major public health burden with a high death toll. Improving the situation requires more systemic vaccination and diagnostic efforts, as well as more effective treatment (23, 24). Current antivirals fail to eliminate HBV infection, and cessation of antiviral therapy leads to viral rebound (25, 26), because cccDNA persists as a stable episome in the nucleus of infected hepatocytes despite treatment. This review will focus on why elimination of HBV cccDNA remains the holy grail for current efforts to find a cure for HBV infection and what potential curative strategies aimed at eliminating or crippling the viral cccDNA would look like.

HBV INFECTION AND PERSISTENCE ARE SUSTAINED BY THE VIRAL cccDNA

HBV entry into human hepatocytes is mediated via the sodium taurocholate cotransporting polypeptide (NTCP), and recent data indicate that it also involves the epidermal growth factor receptor (EGFR) (27, 28). After reaching the nucleus, the HBV genomic rcDNA is converted to cccDNA (Fig. 1), which serves as the transcriptional template for all viral RNAs, including a pregenomic RNA (pgRNA) and, thus, is the molecular basis for establishing and maintaining viral infection (29–31). Viral DNA replication starts with

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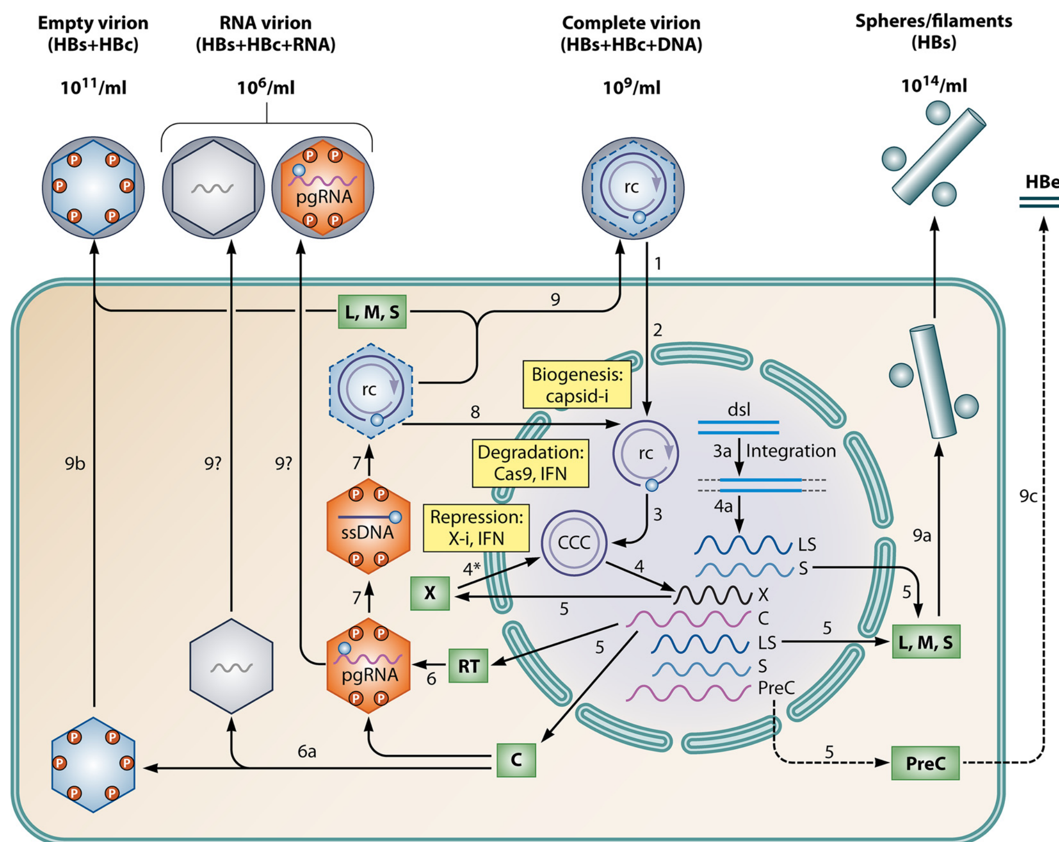

FIG 1 Schematic of hepatitis B virus (HBV) replication cycle. 1. Virus binding and entry into the host cell (large rectangle). 2. Intracellular trafficking and delivery of relaxed circular DNA (rcDNA [rc]) to the nucleus (large circle). 3 and 3a. Conversion of rcDNA to cccDNA (CCC) (3) or integration of the double-stranded linear DNA (dslDNA [dsl]) into host DNA (3a). 4, 4a, and 4*. Transcription to synthesize viral RNAs (wavy lines), including the following: C mRNA for both the core and polymerase (reverse transcriptase [RT]) proteins; LS mRNA for the L envelope protein; S mRNA for the M and S envelope proteins; X mRNA for the X protein, which is transported to the nucleus to stimulate cccDNA transcription (4*); and precore mRNA (PreC) for the precore protein. The C mRNA is also the pgRNA. 5. Translation to synthesize viral proteins. 6 and 6a. Assembly of the pgRNA- (and RT)-containing capsid (6) or, alternatively, empty capsids (6a). 7. Reverse transcription of pgRNA to synthesize the minus-strand ssDNA and then rcDNA. 8. Nuclear recycling of capsids containing progeny rcDNA to form more cccDNA (intracellular cccDNA amplification). 9, 9a, 9b, 9c, and 9?. Envelopment of the rcDNA-containing capsid and secretion of complete virions (9), or alternatively, secretion of empty virions (9b) or spheric and filamentous subviral particles containing HBsAg only (9a). Processing of the precore protein and secretion of HBe (9c). The secretion of putative RNA virions is not yet resolved (9?). The different viral particles outside the cell are depicted schematically as follows, with their approximate concentrations in the blood of infected persons indicated: the complete, empty, or RNA virions are depicted as large circles (outer envelope) with an inner hexagonal shell (capsid) with or without rcDNA (unclosed, double concentric circle) or RNA (wavy line) inside the capsid, respectively, and subviral spheres and filaments as small circles and a cylinder. It is important to point out that the concentrations of all these particles can vary widely between different patients and over time in the same patient. Intracellular capsids are depicted as hexagons, either containing viral pgRNA, minus-strand ssDNA (straight line), or rcDNA or empty. The letters "P" denote phosphorylated residues on the immature capsids (containing ssDNA or pgRNA) or empty capsid. The dashed line of the diamond in the rcDNA-containing mature capsid signifies the destabilization of the mature capsid, which is dephosphorylated. The empty capsids, like mature capsids, are also less stable than immature capsids but, unlike mature capsids, are phosphorylated. The soluble, dimeric HBeAg is depicted as gray double bars. The thin dashed line and arrow denote the fact that HBeAg is frequently decreased or lost late in infection. Boxed letters denote the viral proteins translated from the mRNAs. The filled circle on rcDNA denotes the RT protein attached to the 5′ end of the minus strand (outer circle) of rcDNA, and the arrow denotes the 3′ end of the plus strand (inner circle) of rcDNA. For simplicity, synthesis of the minor dslDNA in the mature capsid, its secretion in virions, and infection of dslDNA-containing virions are not depicted here. Potential therapeutic strategies targeting cccDNA are highlighted in yellow boxes. i, inhibitor. See text for details. (Adapted from *Viruses* [180]).

the assembly of homomultimers of the viral core protein (HBc) into a replication-competent but still immature viral capsid in the cytoplasm, incorporating pgRNA and the viral polymerase as a specific ribonucleoprotein complex. In contrast to conventional retroviruses, the HBV polymerase initiates reverse transcription using its terminal protein domain as a protein primer, resulting in the covalent attachment of the polymerase to the 5′ end of the minus-strand DNA (protein priming)

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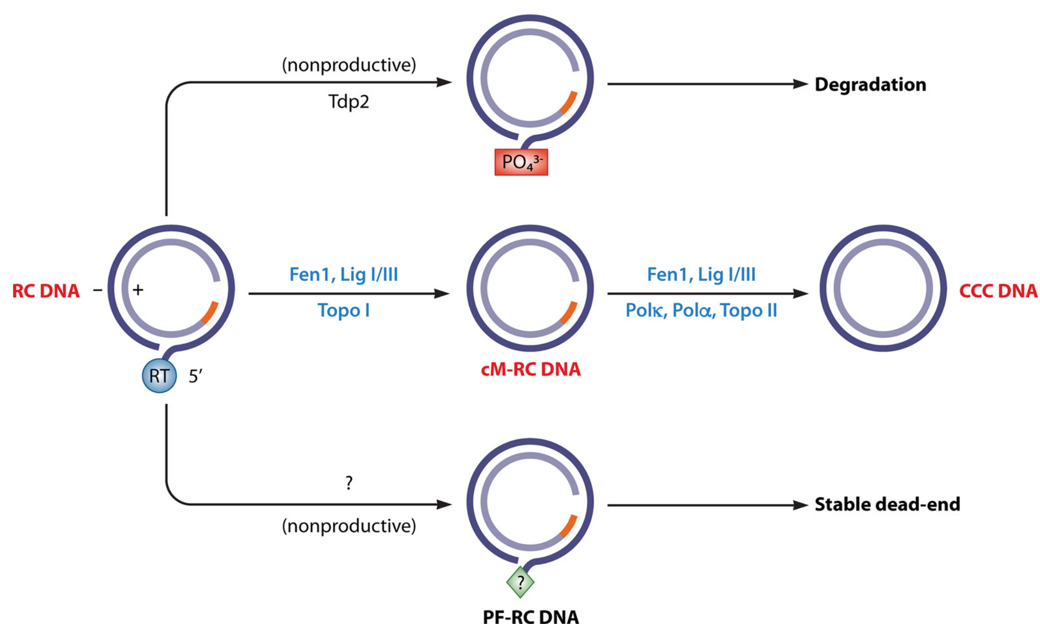

FIG 2 Productive and nonproductive pathways of HBV cccDNA formation. The closed minus-strand rcDNA (cM-RC DNA) (middle row) is considered to be a likely intermediate during cccDNA (CCC DNA) formation. Other protein-free-rcDNA (PF-RC DNA) species (top and bottom rows) are considered to be off-pathway products for cccDNA formation. Putative host factors involved in generating the various PF-rcDNA species and cccDNA are indicated. The question mark in the diamond (bottom) denotes the unknown nature of the 5′ end of the minus strand of this PF-RC DNA species. See text for details. (Adapted from reference 97).

(31, 32). Following protein priming, the reverse transcriptase activity of the HBV polymerase converts pgRNA first to a minus-strand single-stranded DNA (ssDNA) and then to rcDNA (Fig. 1), while the remaining RNA is degraded by its RNAse H activity (15, 31). Mature capsids, i.e., capsids containing rcDNA, can be enveloped and secreted extracellularly as complete virions. Importantly, the newly formed progeny rcDNA in intracellular mature capsids, like that from the incoming virion, can also be delivered to the host cell nucleus and initiate the formation of additional cccDNA molecules, contributing to viral persistence (31, 33–36). In the host cell nucleus, cccDNA is associated with cellular histones and, probably, also other host and viral proteins in a minichromosome (37–39). The chromosomal structure regulates cccDNA transcription and, potentially, also stability.

VIRAL FACTORS INVOLVED IN cccDNA FORMATION

There are five steps that may be involved in the biogenesis of cccDNA from rcDNA (Fig. 2): (i) removal of the covalently attached viral polymerase protein from the 5′ end of the minus strand; (ii) removal of one copy of the terminal redundancy (r) that is present on both ends of the minus strand; (iii) completion of plus-strand elongation; (iv) removal of the RNA oligomer attached to the 5′ end of the plus strand; and (v) ligation of both strands. Whereas the viral polymerase may play a role in completion of the plus strand of rcDNA, current evidence indicates that most if not all of the steps in cccDNA formation are carried out by host cell DNA repair factors.

The HBV genome contains several partially overlapping open reading frames (ORFs) from which the following seven viral proteins are translated (Fig. 1): (i) the HBc protein that forms the viral capsid and the HBe protein processed from a precore protein containing the entire HBc sequence and an additional N-terminal extension; (ii) the small (S), medium (M), and large (L) viral envelope proteins, each of which contains a common S domain and, in the case of M and L, one and two N-terminal extensions, respectively; (iii) the viral polymerase; and (iv) the HBx protein.

The HBc protein is a small (21-kDa) phosphoprotein that plays multiple roles in

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the HBV life cycle, in addition to forming the viral capsid (40, 41). HBc contains an N-terminal domain (NTD) and a C-terminal domain (CTD). The HBc NTD (amino acids 1 to 140), also known as the assembly domain, is essential for capsid formation (42, 43) but also plays a role in pgRNA packaging and reverse transcription (44). The HBc CTD (amino acids 150 to 183) shows nonspecific nucleic acid-binding activity and is critical for pgRNA packaging and reverse transcription (45–47). In addition, the short (9 residues, from position 141 to 149) linker peptide between the NTD and CTD, traditionally thought to be simply a flexible spacer separating the two domains, has recently been shown to play critical roles during multiple stages of virus replication (48).

With respect to the role of HBc in cccDNA formation, current evidence suggests that HBc contributes to cccDNA formation via at least two distinct mechanisms. First, HBc regulates rcDNA nuclear import (Fig. 1) via the nuclear localization signals (NLSs) contained in its CTD (49–51). In addition, mutations in the HBc NTD (e.g., a change of L to A at position 95 [L95A] and K96A) have been shown to increase cccDNA formation, possibly also through effects on nuclear import of rcDNA (52). Second, HBc regulates cccDNA formation by controlling the release of rcDNA from mature capsids (i.e., uncoating or disassembly) (Fig. 1); HBc NTD mutants (e.g., L60A and I126A) that selectively destabilize mature capsids show dramatically enhanced cccDNA levels (52). Since rcDNA requires access to nuclear factors for cccDNA formation, uncoating, like nuclear import of rcDNA, is a prerequisite for cccDNA formation. Uncoating of rcDNA is modulated by multiple determinants of HBc, including the (de)phosphorylation dynamics of its CTD and the structure of the NTD (50, 52–58). Interestingly, an uncoating failure likely contributes to the block to cccDNA formation in mouse hepatocytes and, thus, the species tropism of HBV (59).

The viral envelope proteins, particularly the largest one, L, act as negative regulators of cccDNA formation by directing mature capsids into virions (35, 60–63) instead of retargeting them to the nucleus. Current evidence indicates that the DNA synthesis activity of the HBV polymerase is not essential for any step in cccDNA formation (64–67), although it may contribute to elongating the plus strand in rcDNA.

Therapeutic targeting of viral factors involved in cccDNA formation. Current antiviral therapies with NUCs target the viral polymerase and inhibit reverse transcription. They block synthesis of the viral genomic rcDNA to prevent the formation of mature capsids and the release of infectious progeny virus, thereby diminishing the replenishment of cccDNA through either *de novo* infection or intracellular cccDNA amplification (Fig. 1). However, NUCs cannot target established cccDNA and, thus, fail to cure chronic HBV infection (68).

Given the critical role of the HBV capsid protein in controlling cccDNA formation, it was anticipated that capsid-targeted antivirals, which are currently in clinical development for therapy of chronic hepatitis B (69, 70), may be able to modulate cccDNA formation. Indeed, a number of small-molecule, capsid-targeted compounds have been shown recently to modulate cccDNA formation, potentially by affecting capsid disassembly (71–74). Furthermore, enhanced or premature NC uncoating can trigger host antiviral innate immunity to clear persistent HBV by exposing the viral DNA to trigger the host DNA sensing and defense mechanisms (75–77). Therefore, a better understanding of HBV uncoating will not only help elucidate virus and host control of cccDNA formation and HBV host species tropism but may also reveal novel means of targeting innate immunity for curative therapy of chronic HBV infection.

Host factors involved in cccDNA formation. It is generally agreed that the host cell DNA damage repair machinery recognizes rcDNA as damaged DNA and mediates its conversion to cccDNA (31, 36, 78, 79). cccDNA formation is highly efficient in permissive hosts *in vivo*, as a single rcDNA from the virion is sufficient to establish productive infection in chimpanzees infected with HBV and ducks infected with duck HBV (DHBV) (80, 81). Although continuously improving (82), *in vitro* cell culture systems

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commonly used to study cccDNA formation (83) are unable to recapitulate this high efficiency, implying a certain risk of missing some of the host factors involved.

A few specific host DNA repair factors have recently been identified as potentially playing a role in HBV cccDNA formation (Fig. 2). Tyrosyl DNA phosphodiesterase 2 (Tdp2), which was identified by its ability to remove topoisomerase II (Topo II) from covalent DNA adducts (84), is clearly able to cleave the HBV polymerase from rcDNA, precisely at the phosphodiester bond between the terminal protein and the 5′ end of the minus-strand DNA (85–88). However, Tdp2 is not essential for and may even suppress HBV cccDNA formation, as Tdp2 knockdown or knockout not only did not block but even increased HBV infection and cccDNA formation.

Flap endonuclease 1 (Fen1), known to cleave unannealed 5′ DNA fragments at DNA three-strand junctions (as found on rcDNA near the covalently attached polymerase [Fig. 2]), has recently been reported to play a role in cccDNA formation (89), presumably by cleaving the 5′ repeat (r) fragment from the 5′ end of the minus strand and, possibly, the 5′ RNA oligomer of the plus strand. The host DNA polymerase kappa (Polκ) and a few other host DNA polymerases have recently been identified as host factors contributing to cccDNA formation during HBV infection (67). In contrast, DNA polymerase alpha (Polα) is reported to play a role in cccDNA production via the intracellular amplification pathway, suggesting that cccDNA formation during *de novo* infection and intracellular recycling may use different host polymerases (90). Although these polymerases are presumed to function by completing the plus-strand DNA in rcDNA, there is currently no direct evidence in support of this. Indeed, Polα appears to be needed for minus-strand closing (see below).

Both DNA ligase I (Lig I) and Lig III were recently reported to play a role in cccDNA formation, presumably by ligating either or both strands in rcDNA (91). The only other cellular DNA ligase, Lig IV, is involved in forming defective cccDNA from double-stranded linear DNA (dslDNA), a minor genomic DNA form (Fig. 1) with imprecise junctions, via nonhomologous end joining (NHEJ) (91–93). Topo I and II have also been reported to play a role in HBV cccDNA formation (94). It is not yet clear how Topo I or II facilitates cccDNA formation, although it was suggested two decades ago that Topo I may cleave both strands of rcDNA near the DNA ends (95). Clearly, additional studies are required to verify the role of these host factors in cccDNA formation and to elucidate their mechanisms of action.

The biochemical pathways of cccDNA formation remain to be defined (Fig. 2). Consistent with the removal of the polymerase protein being one of the first steps in cccDNA formation, a protein-free (PF) or deproteinated rcDNA has been identified in established cell lines that support HBV replication (35, 59, 62, 96). However, the fine structure of the PF-rcDNA, especially at the 5′ end of the minus-strand DNA, remains to be characterized, and PF-rcDNA may actually represent multiple related DNA species, some of which may in fact be dead-end processing products from the (polymerase-linked) rcDNA (35, 62, 88, 96), whereas others are true intermediates for cccDNA formation (Fig. 2). Interestingly, a novel form of PF-rcDNA in which the minus strand is closed but the plus strand remains open, the so-called closed minus-strand rcDNA (cM-rcDNA) (Fig. 2), has recently been identified in cultured cells during both HBV infection and intracellular cccDNA amplification (97). This rcDNA processing product is the best candidate to date for an authentic intermediate in cccDNA formation, which would further suggest that minus-strand closing occurs before that of the plus strand during cccDNA formation. Interestingly, Topo I appears to be needed for cM-rcDNA formation and, thus, for the closing of the minus strand, whereas Topo II is more important for the closing of the plus strand (94).

In addition to the enzymatic machinery that carries out the actual DNA repair reactions to form HBV cccDNA from rcDNA, other host factors, including host protein kinases and phosphatases that regulate the HBc phosphorylation state and, therefore, capsid nuclear import and uncoating (98–102), are likely involved in controlling cccDNA formation. However, since the host factors identified so far as being involved in cccDNA

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formation are also involved in a number of essential cellular processes, they appear less promising as therapeutic targets because of potential severe side effects.

Role of HBx protein in cccDNA transcription. HBx is a regulatory protein that has attracted the attention of a large number of investigators, and hence, voluminous literature exists on HBx, most of which may not have been carried out in physiological context (103). HBx is unequivocally required for viral replication (21, 104, 105). In HBV infection, it is essential to initiate and maintain transcription from cccDNA (106). It has been shown to contribute to disease pathogenesis, including contributing to the initial stages of hepatocellular carcinoma indirectly via signal transduction pathways, as well by altering host gene expression (21, 107).

HBx has been localized to both the nucleus and cytoplasm, enabling it to play multiple roles in regulating gene expression and signal transduction (106, 108–110). In the nucleus, HBx binds the cccDNA minichromosomes and exerts its influence epigenetically (111). HBx does not directly bind DNA but may bind to host transcription factors, such as ATF-2 and CREB, that bind cognate sequences of the promoters (112, 113). Among the basal transcription factors, the most notable are the helicase subunits (ERCC2/3) of transcription factor II human (TFIIH), implicated in DNA repair (114). A fraction of HBx associates with mitochondria via a voltage-dependent anion channel (VDAC) protein at the outer mitochondrial membrane (115, 116). Mitochondrial association leads to activation of signal transducer and activator of transcription 3 (STAT3) and nuclear factor κB (NF-κB) via oxidative stress (117). HBx interacts with mitochondrially recruited Parkin protein, an E3 ubiquitin ligase, which then recruits cytoplasmic linear ubiquitin assembly complex (LUBAC) to mitochondria (118). HBV infection can induce bulk autophagy, as well as selective autophagy of mitochondria via HBx (119). Autophagy is required for HBV DNA replication and contributes to persistent infection (120). The translocation of Parkin to mitochondria induced by HBV causes massive ubiquitination of mitochondrial antiviral signaling adaptor (MAVS) and affects its ability to further interferon (IFN) signaling. Mitophagic quality control allows infected hepatocytes to maintain persistent hepatitis, cellular homeostasis, and innate immunity (118, 121).

HBx's involvement with DNA repair pathways, particularly its binding to the DNA damage-binding protein (DDB1), is critical for virus replication (21, 122). Its interactions with the cellular proteasome complex via DDB1 have been characterized in detail (123). Finally, HBx has been shown to localize to the cccDNA minichromosome (111) and function epigenetically by influencing factors like those bound to and regulating heterochromatin (124, 125) and negatively regulating HBV transcription (21). HBx recruits a DDB1-containing E3 ubiquitin ligase to degrade the host restriction factor SMC5/6 to permit HBV transcription from cccDNA, which would otherwise be repressed transcriptionally (126). Although most of these data have largely been obtained by overexpressing HBx, nevertheless, they contribute to our understanding of its role in regulating host cell signaling and gene expression. These studies together confirm the essential role of HBx in initiating and maintaining transcription from HBV (21). Given its central role in the HBV life cycle, in addition to affecting epigenetic transcription and stimulating numerous cellular transduction pathways, targeting HBx emerges as a viable therapeutic strategy.

Host factors affecting cccDNA transcription. Besides its restriction to human or humanoid primate hosts, HBV replicates exclusively in hepatocytes, owing largely to a combination of liver-specific and ubiquitous transcriptions factors that bind to cognate sequences on the HBV promoters and enhancers on cccDNA, conferring liver specificity. Chief among the liver-specific transcription factors are CCAAT-enhancer-binding protein (C/EBP), regulated by protein phosphatase 1 (127), activating transcription factor 2 (ATF-2), hepatocyte nuclear factor 3 (HNF3)/FoxA, HNF1, and in particular, HNF4 (128), as well as the family members of nuclear hormone receptors that include retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), luteinizing hormone receptor (LHR), etc. (112, 129–134). Numerous studies have

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characterized the role of these transcription factors in regulating the liver-specific transcription from promoters and enhancers of HBV genes in different proportions (128, 135–140). Viral transcription is varied in strength and proportional to the synthesis of proteins. For instance, the viral envelope proteins, particularly the S protein, are expressed quite efficiently, as reflected by the robust production of an excess of subviral particles that are secreted to the patients' blood, where they are detected as HBsAg, which has served as a convenient marker for the onset and persistence of hepatitis B. The precore/core proteins are also expressed at high levels, whereas the polymerase protein, HBx, and the L protein are modestly expressed. All HBV transcripts except the one encoding the L protein initiate from clustered sites upstream from the ATGs of the respective ORFs but terminate at a single unconventional polyadenylation site, 5′-UAUAAA-3′ (Fig. 1) (141).

Several cytokines control HBV transcription through liver-enriched transcription factors. Transcriptional activity of the HBV genome in livers of HBV-transgenic mice is reduced upon infection with a DNA virus or treatment with poly(I·C)-inducing alpha interferon (IFN-α) and IFN-β (142). IFN-induced tripartite motif 22 (TRIM22) inhibits HBV core promoter activity and, thus, HBV gene expression and replication *in vitro* and *in vivo* (143). Interleukin-4 (IL-4) suppresses C/EBPα and shows a direct antiviral effect on HBV by reducing the activity of HBV surface promoter II and the core promoter (144). IL-1β regulates cccDNA transcription via hepatocyte dedifferentiation, resulting in loss of HNF4α (145). IL-6 suppresses the expression of HNF1α and HNF4α, two major transcription factors determining HBV promoter activity, by activating mitogen-activated protein kinases Jun N-terminal protein kinase (JNK) and extracellular signal-regulated kinase (ERK) (146). Similarly to IL-6, transforming growth factor β (TGF-β) represses HNF4α expression (147). Thus, IL-6, IL-1β, and TGF-β target HBV transcriptional activity and HBV replication through modulating the expression of the essential transcription factor HNF4α, while IL-4 seems to repress C/EBPα (148). IFNs not only affect transcriptional activity but also destabilize HBV RNAs, e.g., by exposing cleavage-sensitive RNA motifs (149, 150). In addition, IFN-stimulated gene 20-kDa protein (ISG20) is an exonuclease that degrades viral transcripts (151). For therapeutic purposes, however, only IFN-α and IFN-β are licensed, and IFN-λ was evaluated only in a small number of patients (152); all other cytokines unfortunately have too many side effects.

Epitranscriptomic and epigenetic control. The most abundant RNA modification in mammalian cells is the methylation of adenosine at the nitrogen-6 position (N6 methyladenosine [m^6A]), with implications in various processes, including immune response, development, differentiation, cellular homeostasis, etc. (153). HBV transcripts are m6A modified, which confers instability to RNA and regulates translation (154). More importantly, this m^6A modification positively regulates the reverse transcription of encapsidated pgRNA and affects the turnover of cytoplasmic viral RNAs differentially, thus playing a negative role in overall HBV gene expression (154). The epitranscriptomic N6 methyladenosine modification adds a new layer of complexity in the regulation of viral gene expression and may open up new therapeutic opportunities.

In contrast to RNA methylation, epigenetic modification of DNA has long been established (155). cccDNA forms a minichromosome and, thus, is subject to epigenetic modification, which refers to modification of a DNA without changes in its sequence that are inheritable from mother to daughter cells. Epigenetic regulation mechanisms include but are not limited to DNA methylation, histone modifications, chromatin remodeling, and noncoding RNA interference. As HBV cccDNA exists in the nucleus as multiple copies of histone-decorated minichromosomes, all these regulatory mechanisms may influence HBV gene expression and, indeed, have been described to influence HBV replication and, potentially, even persistence (156). Accordingly, epigenetic therapy has long been proposed as a therapeutic option to target cccDNA. A number of drugs against epigenetic targets have been developed for cancer therapy and may be repurposed for HBV therapy. Obviously, the goal of an epigenetic therapy

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is to silence cccDNA and, thus, to terminate HBV gene expression, antigen release, and replication (157). While drug development targeting methyltransferases is still under way, 5-azacytidine and 5-aza-20-deoxycytidine are examples of FDA-approved DNA methyltransferase (DNMT) inhibitors. They are, however, hepatotoxic and thus not suited for therapy of viral hepatitis. Alternative developments are needed, including, e.g., that of epigallocatechin-3-gallate, which has been described to have anti-HBV effects (157). Epigenetic modifier drugs, however, are unlikely to eliminate HBV and bear the risk of unwanted side effects, since they will also influence host cell gene expression.

A number of cytokines have been described as affecting the epigenetic status of HBV cccDNA. Belloni et al. showed that IFN-α inhibits HBV replication by decreasing transcription of viral RNA from the HBV cccDNA minichromosome in cell cultures and in humanized mice (158). IFN-α treatment reduced the binding of the transcription factors STAT1 and STAT2 to the IFN-sensitive response element on active cccDNA, resulting in hypoacetylation of cccDNA-bound histones, and recruited transcriptional corepressors, including histone deacetylase 1 (HDAC1), to cccDNA (158). In HBV-infected primary human hepatocytes, IFN-α represses HBV by reducing active histone marks on the cccDNA minichromosome (39). Interestingly, a small-molecule epigenetic modulator, C646, that specifically inhibits p300/CREB-binding protein (CBP) histone acetyltransferases recapitulated the effect of IFN-α (39). IL-6 reduced cccDNA-bound histone acetylation and led to redistribution of STAT3 from the cccDNA to cellular IL-6 target genes, and tumor necrosis factor alpha (TNF-α) degraded nuclear factors (159). This induced a rapid decrease of all HBV RNA transcripts without affecting cccDNA integrity (160). IL-1β was able to silence cccDNA transcription by inducing inhibitory NF-κB that binds to cccDNA (145).

IMMUNE CLEARANCE OF HBV cccDNA

The efficiency of the HBV-specific immune response in clearing or at least fully controlling HBV cccDNA becomes evident during resolution of an acute infection, during which almost all hepatocytes become infected (161, 162). Ninety-five percent of immunocompetent adults resolve an acute HBV infection, displaying an efficient B cell response characterized by high-level neutralizing anti-HBs antibodies, as well as a robust T cell response with CD4 and CD8 T cells capable of producing antiviral cytokines and killing infected hepatocytes. The dichotomy between patients that resolve an acute infection and those with chronic HBV infection can be correlated with both the magnitude and function of the host immune response (summarized in reference 163).

A significant part of the immune control during acute HBV infection is due to hepatocyte killing (164). There is a large body of evidence that T cells are crucial players in this context. Viral clearance during acute HBV infection is thought to be mediated by cytotoxic CD4 and CD8 T cells (165, 166) that can directly recognize and kill infected hepatocytes. Patients that control HBV after long-term nucleoside analogue therapy have T cell frequencies similar to those of patients who resolved acute infection (167).

Immune cells, however, can also control the virus in a noncytolytic fashion via the secretion of cytokines and other immune mediators. In HBV-infected chimpanzees, noncytolytic antiviral mechanisms contribute to viral clearance by purging HBV replicative intermediates from the cytoplasm but also reduce cccDNA before the peak of T cell-mediated hepatocyte killing (161). For the noncytolytic control of HBV by T cells in HBV-transgenic mice, which have no cccDNA, antiviral cytokines like IFN or TNF-α, secreted from T cells and other sources, play an essential role (168). Interestingly, the same cytokines were recently demonstrated to also contribute to purging of cccDNA in HBV infection (169, 170).

IFN-β or IFN-γ treatment eliminates pgRNA-containing capsids in murine cells in cell culture (171). HBV-transgenic mice treated with poly(I·C), which induces an IFN-α/β response, rapidly depleted cytoplasmic pgRNA-containing capsids, while HBV mRNA

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and its translational status remained unchanged, indicating that IFN signaling prevented the formation of replication-competent pgRNA-containing capsids in infected hepatocytes (172). Thus, depending on the experimental system, the IFN-induced cellular antiviral response seems able to inhibit transcription, to destabilize HBV transcripts, and to selectively accelerate the decay of replication-competent HBV capsids in a proteasome-dependent manner.

High doses of cytokines are able to trigger noncytolytic purging of cccDNA from infected primary human hepatocytes or HepaRG cells (173). IFN-α activates the APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like cytidine deaminase) family member APOBEC3(A3)A (A3A) that utilizes HBc to get access to cccDNA, lymphotoxin-β receptor agonists induce the nuclear deaminase APOBEC family member A3B in an NF-κB-dependent fashion (173), and type I and III interferons, as well as TNF, induce both deaminases (169, 174). Deaminated cccDNA becomes prone to degradation by nucleases that are also IFN regulated. Treatment of deaminated cccDNA with base excision repair enzymes and apurinic/apyrimidinic endonuclease leads to cccDNA decay (173). The upregulation of A3A was confirmed in liver biopsy samples from IFN-α-treated patients and chimpanzees. Interestingly, it can activate the same pathway, resulting in cccDNA purging (173). A clinical study in a limited number of patients found A3A and base-excision repair gene expression to be upregulated in patient blood and liver biopsy samples after pegylated IFN-α (PEG-IFN-α) therapy, and this correlated with antiviral effects of IFN therapy (175).

TGF-β could also induce cccDNA deamination and degradation in hepatocytes via activation-induced cytidine deaminase (AID) (176), which has been described to mediate degradation of duck HBV (DHBV) cccDNA (177). The effect elicited by TGF-β was abrogated when AID or the activity of uracil N-glycosylase (UNG) was blocked, indicating that AID-mediated deamination and the excision of uracil by UNG act in concert to degrade HBV cccDNA (176). Similar to the case for A3A, the interaction between AID and viral cccDNA is mediated by HBc.

These observations render the noncytolytic control of cccDNA and, even more so, the purging of cccDNA by cytokines an interesting antiviral mechanism that may be exploited by, e.g., Toll-like receptor, retinoic acid-inducible gene I (RIG-I), or stimulator of interferon genes (STING) agonists that induce these cytokines. However, the question of whether a cure of HBV is possible through this mechanism remains open. In most studies, cccDNA levels were reduced, but cccDNA was never completely eliminated or controlled without the cytotoxic activity of immune cells. It may therefore also be an escape mechanism of the virus from overwhelming immune responses.

CONCLUSIONS

Despite the recent advances in understanding the HBV life cycle and the existence of a prophylactic vaccine and of antivirals that efficiently suppress virus replication, chronic hepatitis B remains a major public health burden. The remaining risk of developing hepatocellular carcinoma (HCC) despite antiviral therapy with nucleos(t)ide analogues (NUCs) and the potential risks of severe side effects and viral drug resistance with life-long therapy point to the clinical importance of developing novel, curative therapies. Although complete elimination of the nuclear cccDNA remains elusive, major efforts are directed toward developing finite therapies that clear or silence HBV cccDNA. A clear understanding of the basic biology of cccDNA, i.e., biogenesis, turnover, epigenetic regulation, and template activity, is required to develop therapies that have the potential to achieve this goal. Along the same line, the immunological clearance and control of cccDNA is an area of high importance and must be explored to all possible limits. Advances in genome editing, particularly the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) technology, may allow the destruction of preexisting cccDNA (Fig. 1) (178). So far, however, effective delivery is lacking and potential host genome damage is a major concern. Convenient animal models and efficient infection systems will be extremely helpful in all these endeavors (83). Finally, there is an urgent need to develop sensitive and

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standardized assays to measure cccDNA and convenient and reliable surrogate markers to monitor intrahepatic cccDNA levels and transcriptional activity in order to guide therapy using current antivirals, as well as the development of novel cccDNA-targeted drugs (24, 179). Current foci of interest in this regard include HBV RNA, HBeAg, HBcAg, HBcrAg, etc., which are readily detectable in the blood of infected patients (Fig. 1).

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