VIROLOGICA SINICA 2017, 32 (6): 454–464
DOI: 10.1007/s12250-017-4009-4

REVIEW

Recent advances in the study of hepatitis B virus covalently closed circular DNA

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Chronic hepatitis B infection is caused by hepatitis B virus (HBV) and a total cure is yet to be achieved. The viral covalently closed circular DNA (cccDNA) is the key to establish a persistent infection within hepatocytes. Current antiviral strategies have no effect on the pre-existing cccDNA reservoir. Therefore, the study of the molecular mechanism of cccDNA formation is becoming a major focus of HBV research. This review summarizes the current advances in cccDNA molecular biology and the latest studies on the elimination or inactivation of cccDNA, including three major areas: (1) epigenetic regulation of cccDNA by HBV X protein, (2) immune-mediated degradation, and (3) genome-editing nucleases. All these aspects provide clues on how to finally attain a cure for chronic hepatitis B infection.

KEYWORDS **hepatitis B virus (HBV); covalently closed circular DNA (cccDNA); HBx; immune-mediated; genome-editing nucleases**

INTRODUCTION

Hepatitis B virus (HBV) replication cycle

HBV is the type member of the *Hepadnaviridae* and its replication cycle starts with binding of the PreS1 domain (amino acids 8–18) of the large envelope protein to the sodium taurocholate cotransporting polypeptide (NTCP) receptor (Yan et al., 2012). Infectious virions contain a relaxed circular DNA (RC-DNA), and the RC-DNA is converted into covalently closed circular DNA (cccDNA) in the host cell nucleus. Serving as a transcription template, cccDNAs are transcribed into several genomic and subgenomic RNAs by cellular RNA polymerase II. Of these RNA transcripts, the pregenomic RNA (pgRNA) is further reverse transcribed by the reverse transcriptase (RT) activity of the P protein into a new RC-DNA genome (Feng and Hu, 2009). Matured RC-DNA-containing nucleocapsids are subsequently enveloped and finally released from host hepatocytes as progeny virions, or re-enter into the nucleus supplementing the cccDNA reservoir (Beck and Nassal, 2007) (Figure 1A).

Received: 28 April 2017, Accepted: 27 November 2017,
Published online: 21 December 2017
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HBV cccDNA

cccDNA is a mini-chromosome in the infected cell nucleus and is the key element in the establishment of a persistent infection. Upon successful infection, the RC-DNA is converted into cccDNA, which is highly stable and acts as a virus transcription template. For viral RNA transcription, cccDNA is transcribed to pgRNA and other viral mRNAs. There are 3–50 copies of cccDNA per infected cell. The number of copies of the cccDNA decreases during the process of host cell division. So, recycling of the new RC-DNA to the nucleus occurs in order to replenish the relatively stable cccDNA copy number (Figure 1A). There are three main steps (Figure 1B) in the RC-DNA to cccDNA conversion: (1) P protein that is covalently linked to the 5′ end of the (–)-DNA is removed; (2) the 5′ end of the (+)-strand consisting of an RNA oligonucleotide is removed; (3) both strands are covalently ligated. To date, the mechanism of RC-DNA

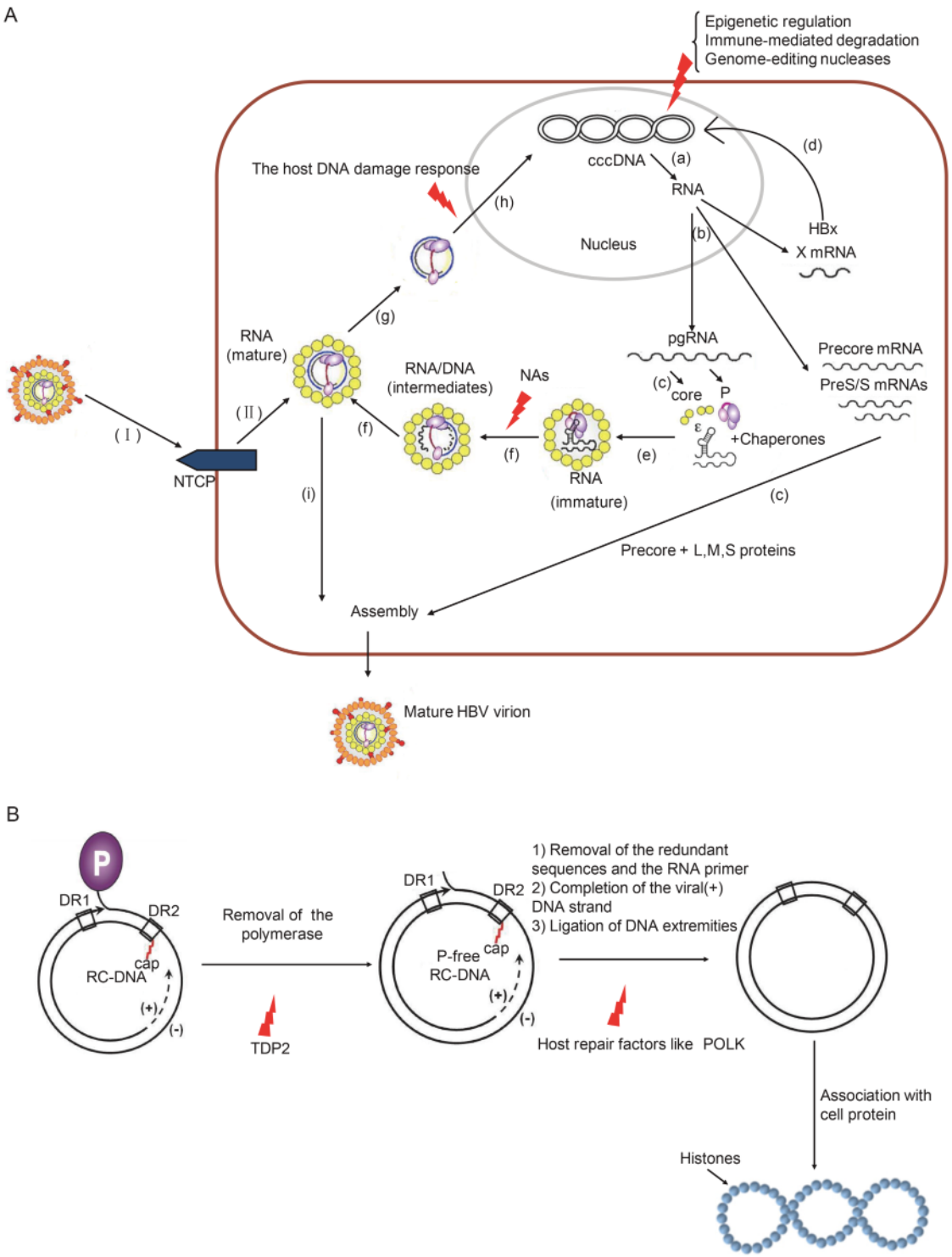

Figure 1. (A) The HBV life cycle (modified from Nassal, 2015; *Gut*; with the author's permission) and the corresponding target interfering strategies (red). I. Attachment and entry. II. Uncoating. (a) Transcription of viral RNAs. (b) RNA nuclear export. (c) Translation. (d) Epigenetic regulation of cccDNA by HBx. (e) Encapsidation with co-packaging of P and pgRNA. (f) Negative strand synthesis (inhibited by nucleoside analogues), pgRNA degradation and positive strand synthesis. (g) Nuclear transport. (h) The conversion of RC-DNA to cccDNA. (i) Envelopment of mature RC-DNA containing nucleocapsids. L, large; M, middle; NA, nucleoside analogue; NTCP, sodium taurocholate cotransporting polypeptide; S, small. (B) The conversion of RC-DNA to cccDNA and the targeting strategy in this process.

transformation into cccDNA is little understood (Nassal, 2015). A study reported that a "P-free RC-DNA" may be the intermediate of RC-DNA conversion into cccDNA (Gao and Hu, 2007). According to Guo *et al.*, P-free (or deproteinized) RC-DNAs are distributed almost equally between the cytoplasm and nucleus (Guo et al., 2007). Other studies showed more deproteinized RC-DNA in the nucleus than in the cytoplasm, which was perhaps due to nuclease treatment of cytoplasmic lysate before Hirt extraction. Guo *et al.* showed that inhibiting the nuclear importation machinery resulted in accumulation of deproteinized RC-DNAs in the cytoplasm and the reduction of such DNAs in nucleus, which suggested that the deproteinized RC-DNA is a functional precursor of cccDNA formation (Guo et al., 2010).

In fact, a large amount of data show that there is a close relationship between viral replication and the intracellular repair system (Lilley et al., 2007). The formation of cccDNA requires multiple enzymatic activities (Figure 2). It has been proven that tyrosyl-DNA phosphodiesterases 2 (TDP2) can remove P proteins from authentic HBV and duck hepatitis B virus (DHBV) RC-DNAs *in vitro* (Königer et al., 2014). The gap requires filling-in by a DNA polymerase. DNA polymerase κ is a key cellular factor involved in cccDNA formation (Qi et al., 2016). HBV RC-DNA, with incomplete incision double chain structure, is likely to be the substrate of the 5′-flap endonuclease (FEN1). As a structurally specific nuclease, FEN1, can remove the free ends of the 5′ single-stranded nucleic acids and is an important member of the DNA repair system (Wood et al., 2001). In addition, it was found that DNA repair system proteins such as tyrosyl-DNA phosphodiesterases (TDPs), DNA polymerases (Qi et al., 2016; Königer et al., 2014), and DNA ligases are involved in this process (Haitao Guo, personal communication, in 2016 International HBV Meeting).

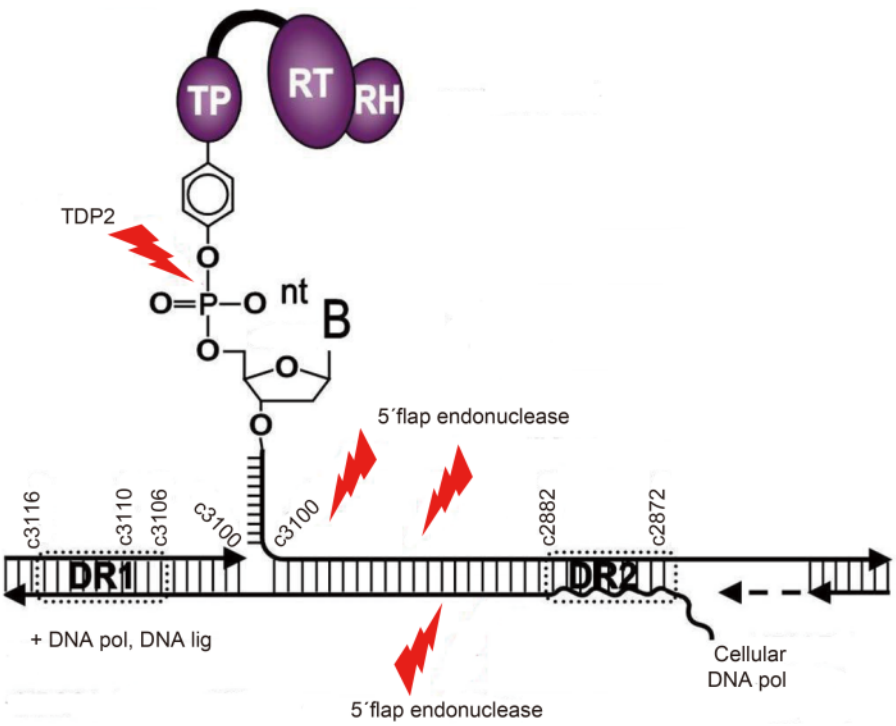

Figure 2. A detailed view of the 5′ ends of (–)-DNA (upper strand) and (+)-DNA is shown. The distance between DR1 and DR2 is not drawn to scale. The (–)-DNA end forms a 5′ flap structure, including the linked P protein and a 10 nt terminal redundancy. P is removed by TDP2, followed by a downstream incision by a 5′ flap endonuclease, and repair synthesis and ligation; cleavage exactly after the 5′ proximal nt c3100 (c for complement to nt 3100 in the (+)-sense) would allow direct ligation without new DNA synthesis. Like 5′ flap endonucleases in TOP1cc repair, 3′ endonucleases may play a role despite their seemingly improper substrate specificity (e.g. acting as 5′ flap endonuclease by association with a different partner, or providing an accessible upstream 3′ OH end to start displacement synthesis). The (+)-DNA 5′ end with the bound RNA primer is topologically equivalent; however, no protein “processing” (TDP2 or proteolysis) would be required. The incomplete (+)-DNA 3′ end can be filled-in by cellular DNA polymerase (Qi et al., 2016). The involvement of cellular DNA ligases is definite, and of end-polishing enzymes is possible also.

Cellular and animal models

Few experimental *in vitro* and *in vivo* models for the study of HBV cccDNA exist. At present, three kinds of *in vitro* cell models are available: primary tupaia hepatocytes (PTH), primary human hepatocytes (PHH) and differentiated HepaRG (dHepaRG). These cell types can be applied for HBV infection. In infected cells, cccDNAs are produced and serve as templates for HBV replication. However, these models have their own limitations that need to be improved under special experimental conditions (Lucifora and Protzer, 2016). In subsequent studies, some new cell lines were developed. HepAD38 is a tetracycline-inducible HBV stable-transfected cell line (Ladner et al., 1997). The expression of pgRNAs is inhibited by tetracycline. Once the tetracycline is removed into the extracellular supernatant, viral pgRNAs in the cells are expressed again. Unlike HepG2.2.15, in which there is no detectable cccDNA during HBV replication, HepAD38 cccDNAs could be found within cellular nuclei, which is similar to the authentic viral life cycle *in vivo*. In addition, due to its high replicative levels, HepAD38 is widely used for the screening of antivirals (Ladner et al., 1997). Zhu Haizhen's group established a new liver cancer cell line, HLCZ01, which supports the entire life cycle of HBV and hepatitis C virus (HCV) replication, providing a new platform for the study of cccDNA (Yang et al., 2014). Furthermore, Yamanaka and colleagues reported that pluripotent stem cells could be *in vitro* induced into hepatocyte-like cells (HLCs) in which a panel of transcription factors, which usually are found only in adult-derived hepatocytes, were expressed (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006). The HLCs have been shown to be self-renewing in culture, remain genetic stability (Si-Tayeb et al., 2010; Touboul et al., 2010; Schwartz et al., 2014) and are susceptible to HBV infection (Schwartz et al., 2014). Recent research revealed that the HBV functional receptor is NTCP (Yan et al., 2012). A NTCP-transduced *in vitro* infection model, HepG2-NTCP and Huh7-NTCP cell lines, thus, has been applied to study HBV infection and cccDNA formation (Hayes and Chayama, 2016; Ni et al., 2014; Qi et al., 2016; Yan et al., 2012).

In addition to the above mentioned *in vitro* HBV infection models, there are *in vivo* models for studying HBV cccDNA, but these are still limited. Animal models of HBV replication, such as transgenic mice, human liver chimeric mice, the woolly monkey model and the chimpanzee model have been reported. Even though HBV transgenic mice offer many possibilities for performing preclinical antiviral testing *in vivo*, the cccDNA is not built within the murine hepatocytes, whereas transcriptionally active cccDNA in human liver chimeric mice was reported (Allweiss and Dandri, 2016). However, the technique for the construction of chimeric liver is expensive and time-consuming. Chimpanzees have been proven to be an invaluable model for investigations on HBV-driven disease pathogenesis and the testing of novel antiviral therapies, as well as having detectable cccDNA in infected livers (Wieland, 2015). Although they mimic a natural human HBV infection to the closest degree, their large-scale application has been restricted, not only due to the cost, but also because of the demanding ethical concerns (Table 1).

Recently, a minicircle HBV cccDNA was engineered with a Gaussia Luciferase reporter (mcHBV-GLuc cccDNA), which serves as a substitute for the detection of cccDNA activity. This model is independent of HBV infection and can be used to study the biology of cccDNA and to screen anti-cccDNA drugs (Li et al., 2016). There is a similar, but different, cccDNA system. A recombinant cccDNA (rcccDNA) produced by using minicircle technology resembles closely its wild-type counterpart, both in structure and function. It is a convenient technology to produce a large quantity of rcccDNA as a surrogate for wild-type cccDNA for investigating HBV biology (Guo et al., 2016). The group of Deng Qing produced a rcccDNA that established HBV persistence with sustained liver injury in immunocompetent mice. The HBV genome was engineered with a Cre/LoxP intron, which could be distinguished using polymerase chain reaction (PCR). This model system provides an innovative approach for testing antivirals directly targeting HBV cccDNA (Qi et al., 2014). Through minicircle technology, HBV circle, a rcccDNA, was easily generated and extracted from a genetically engineered *Escherichia coli* strain. Upon transduction of mouse liver through hydrodynamic injection, HBV circle formed cccDNA-like molecules *in vivo* and persistent virus replication was successfully established. The HBV circle is a close mimic of cccDNA and it represents a novel tool for addressing HBV cccDNA issues and for anti-HBV drug discovery (Yan et al., 2017).

At present, the currently available infection-dependent systems still have some technical limitations, for example, proper HepaRG differentiation requires a long and complex process. However, the infection-independent cccDNA model system is becoming a useful tool for finding cccDNA-relevant host factors involved in the intracellular steps of hepadnavirus replication and for screening chemical inhibitors of cccDNA formation. Minicircle technology is able to produce cccDNA-like molecules, but *de novo* biogenesis of cccDNA cannot be investigated through this system. Thus, more robust infection-susceptible cellular and animal models need to be found.

THE MOLECULAR MECHANISM OF cccDNA FORMATION AND TARGET STRATEGY

Conversion of RC-DNA to cccDNA

The unique notched ring structures of RC-DNA are similar to damaged structures in intracellular DNAs, the

Table 1. A comparison of the advantages and disadvantages for cellular and animal models with detectable cccDNAs

Models		Advantages	Disadvantages
Cell models	PTH/PHH	-intracellular environment close to that *in vivo*	-poor accessibility; -limited cell numbers; -lot-to-lot variation; -genetic manipulation impossible
	HepaRG	-homogeneous source	-license protected, which limits wide application; -induction of differentiation time-consuming and complicated
	HepAD38	-homogeneous source; -genetic manipulation possible	-intracellular environment quite different from that *in vivo*
	HLCZ01	-homogeneous source; -genetic manipulation possible	-not yet widely used
	NTCP-HepG2/Huh7	-homogeneous source; -genetic manipulation possible	-intracellular environment quite different from that *in vivo*
Animal models	Transgenic mice	-wide applications; -simple techniques	-no detectable cccDNAs in liver; -no associated hepatitis disease for mouse
	Human liver chimeric mice	-cccDNAs detectable in liver	-high costs; -unstable results; -low cccDNA copy numbers; -complicated techniques
	Woolly monkey	-cccDNAs detectable in liver	-high costs; -long time periods for study; -ethical concerns
	Chimpanzee	-cccDNAs detectable in liver; -situation *in vivo* similar to that in human	-high costs; -long time periods for study; -ethical concerns

latter induce DNA repair responses that lead to the notched DNAs being restored to normal double chains. In addition, host-derived topoisomerase (TOP), which is called "cutting complex" and is an important member of the host DNA repair system, has a common characteristic that its acting substrate is the protein-DNA connected tyrosyl-DNA-phosphodiester (TDP) bond. These data suggest that the host cell DNA repair system is involved in transformation of RC-DNA to cccDNA.

In the process of RC-DNA conversion into cccDNA, the first need is to eliminate the P protein, so that RC-DNA is converted into P-free RC-DNA. The most striking feature of RC-DNA is the covalent binding of the 5′ terminal of the negative chain to P protein with a phosphodiester linkage, which is similar to the tyrosyl-DNA-phosphodiester bond of the TOP cleavage complex. TDPs repair DNA adducts (Pommier et al., 2014). Recent studies have shown that human TDP2, chicken TDP2 and yeast TDP1 could cleave specifically hepadnavirus-adapted Tyr-5′-DNA model substrates and release P proteins from authentic HBV and DHBV RC-DNA *in vitro* (Königer et al., 2014). These findings are indicative that TDP2 possesses the ability to remove P protein. TDP2 acts in the process of conversion of RC-DNA to cccDNA by cracking the phosphodiester linkage to remove P protein, but in TDP2 knockdown cells, P protein would be removed through other as yet unknown ways, and subsequently transformed into cccDNA. In this case, the conversion process becomes slower rather than being completely blocked. In summary, the role of TDP2 in HBV cccDNA formation remains controversial (Figure 1B) and some results are indicative that TDP2 can remove the RT which is covalently linked to the 5′ end of the HBV minus strand DNA *in vitro*, but this protein might not be indispensable for cccDNA formation *in vivo* because human HBV was still able to infect these TDP2 knockout cells (Cui et al., 2015). It was assumed dsL-DNA may be circularized by the non-homologous end-joining (NHEJ) DNA repair pathway into cccDNA-like molecules (Nassal, 2015). Error-prone classical NHEJ (c-NHEJ), by underlying a major break repair mechanism in single-strand DNA breaks, is likely to be involved

in the DNA damage response in regard to cccDNA formation (Schreiner and Nassal, 2017).

Recent studies in HepG2-NTCP have shown that DNA polymerase κ (POLK) (Figure 1B) is a key cellular factor involved in cccDNA formation (Qi et al., 2016). At 24 hours post-infection, formation of cccDNA could be observed. A few days later, cccDNA copy numbers gradually increased and finally remained at a relatively stable level, with the mean copy number in each infected cell approximately 3. Different from DHBV, the efficiency of the intracellular complement pathway of cccDNA in HBV cells does not seem high. During the early stage of infection, RC-DNAs were converted into cccDNAs, whose accumulation formed a cccDNA pool in the hapatocytic nucleus. Further genetic experiments showed that the initial formation of HBV cccDNA does not rely on the viral DNA polymerase. Fifteen different kinds of DNA polymerases in host cells were screened by targeted RNAi assay. It was found that DNA polymerase κ, DNA polymerase λ (POLL) and a DNA polymerase η (POLH) are involved in the formation of cccDNA. Knockdown of POLK is the most effective way to reduce the copy number of cccDNA. After knockout of POLK expression by CRISPR/Cas9 editing in HepG2-NTCP cells, synthesis of cccDNA was dramatically decreased. After overexpression of POLK in these POLK-knockdown HepG2-NTCP cells, production of cccDNA was restored again. Thus, DNA polymerase κ is a key factor during the formation of cccDNA. For clinical anti-HBV treatment, in order to reduce the synthesis of cccDNA, we need to block virus *de novo* infection (Qi et al., 2016). POLK acts as a functional part in the nucleotide excision repair (NER) pathway by filling the gap produced upon excision of damaged nucleotides (Oqi and Lehmann, 2006). Because cellular DNA polymerases need to be combined with other DNA repair proteins, such other proteins in the NER pathway may also effect HBV cccDNA formation (Qi et al., 2016). For instance, HBV may hijack cellular endonuclease (e.g. XPG) or exonuclease (e.g. Exol) to cut the capped RNA primer, and subsequently POLK or other cellular DNA polymerases are responsible for filling the gap using the minus-strand DNA as a template. POLK has been shown to work together with DNA polymerases δ (POLD) to fill in single-stranded DNA gaps (Ogi and Lehmann, 2006; Ogi et al., 2010).

In addition, Prof. Haitao Guo's group found that the host DNA ligases, specifically Lig1 and Lig3, play a role in hepadnavirus cccDNA formation, which confirmed the involvement of the host DNA repair machinery (Figure 1A) in cccDNA formation (Haitao Guo, personal communication, in 2016 International HBV Meeting).

Epigenetic regulation of cccDNA

Some progress is being made in regard to the study of the epigenetic regulation of cccDNA. It represents an alternative therapeutic option to target cccDNA by epigenetic silencing. Epigenetic alterations of cccDNA, such as histone modification and DNA methylation, are involved in mediating the transcriptional activity of HBV cccDNA (Figure 1A).

HBV X protein (HBx) affects the epigenetic function of nuclear HBV cccDNA, mainly through major regulatory enzymes involved in the formation of cccDNA, including P300, CBP, the histone acetyltransferase P300/CPB-associated factor (PCAF), histone deacetylase 1 (HDAC1), and hSirt1. Histones H3 and H4 are highly acetylated after cccDNA binding to HBx (Belloni et al., 2009). HBx is known as the central regulator for HBV replication and is associated with the cullin4–damage-specific DNA binding protein 1(CUL4–DDB1) ubiquitin ligase through an H-box motif (Guo et al., 2014). After HBV infection, SETDB1-mediated H3 di- and trimethylation (H3K9me3) and heterochromatin protein 1 factors (HP1) induced HBV cccDNA transcriptional silencing by regulating the nuclear chromatin structure. HBx can alleviate this process and allow the establishment of active chromatin (Rivière et al., 2015). Smc5/6 complex is a restriction factor selectively blocking extrachromosomal DNA transcription (Decorsière et al., 2016). Recent reports are indicative that a primary function of HBx is to degrade the Smc5/6 via $CRL4^{HBx}$ E3 ligase (Murphy et al., 2016). Smc5/6 is an antiviral restriction factor that suppresses HBV transcription when localized to Nuclear Domain 10 (ND10) without inducing a detectable innate immune response and is counteracted by the HBx shortly after infection (Niu et al., 2017). A recent study demonstrated that protein arginine methyltransferase 5 (PRMT5) restricts HBV replication via epigenetic repression of cccDNA transcription and interference with pgRNA encapsidation (Hong et al., 2017).

DNA methylation usually occurs at the 5′ position of the cytosine ring within CpG dinucleotides by DNA methyltransferases (DNMTs) in mammalian cells. This process is generally associated with transcription silencing (Schubeler, 2015). It has been reported that increased expression of DNMTs facilitates the methylation of the viral genome to suppress HBV replication *in vitro* (Vivekanandan et al., 2010), indicating that DNMTs could be utilized to inhibit cccDNA transcription. DNMT-based epigenetic therapy may also evoke the innate immunity in HBV-infected cells to enhance the suppression of HBV replication (Hong et al., 2017).

In addition, HBV replication and transcription are inhibited by inflammatory cytokines (tumor necrosis factor-α (TNFα), lymphotoxin β (LTβ)) and interleukin-6 (IL6). The IL6 repressive effect on HBV replication is mediated by a loss of hepatocyte nuclear factor 1α (HNF1α) and hepatocyte nuclear factor 4α (HNF4α)

binding to the cccDNA, and a redistribution of signal transducers and activators of transcription 3 (STAT3) binding from the cccDNA to IL6 cellular target genes (Palumbo et al., 2015).

Immune-mediated degradation

For cccDNA, an interesting issue is whether it can be degraded by immune attack during chronic infection, just like the situation in acute infection. Recently, induction of intrahepatic antiviral immune responses with high doses of interferon-α (IFN-α) or lymphotoxin β receptor (LTβR)-agonists was able to trigger noncytolytic degradation of cccDNA (Figure 1A) from infected cells *in vitro*. Activation of nuclear deaminases, such as APOBEC3A and APOBEC3B, resulted in cccDNA deamination and a significant reduction of cccDNA. IFN-α upregulated the expression of APOBEC3A. A superagonistic tetravalent bi-specific antibody (BS1) and a bivalent anti-LTβR monoclonal antibody (CBE11) activated the expression of LTβR, which in turn increased the expression of APOBEC3B. Connecting through the core interactions, APOBEC3A and APOBEC3B acted on cccDNA jointly changing it to a deaminated state, which destroyed the integrity of the cccDNA. After excision of uracil by uracil DNA glycosylase (UNG) in AP endonucleases, cccDNA was degraded (Lucifora et al., 2014). A later study showed that the level of cccDNA in cancerous tissues was significantly lower than that in non-cancerous tissues. In cancerous tissues, APOBEC3B was the only up-regulated factor both in transcription and in protein-expression level, implying that APOBEC3B may cause cccDNA editing and subsequent degradation in cancerous tissue (Luo et al., 2016). UNG inhibition and the expression of APOBEC3G reduced synthesis of cccDNA. UNG excised uracil residues from the viral genomes during the nuclear cccDNA forming process or after that. At the same time, activation of the host base excision repair pathway reduced APOBEC3-induced hypermutation of HBV genomes (Kitamura et al., 2013).

Genome-editing nucleases

So far, all clinically applied antiviral therapies only inhibit HBV DNA synthesis in the cytoplasm of infected hepatocytes, but are not able to destroy nuclear viral cccDNA. Using gene-editing technology to remove cccDNA represents a novel strategy.

In a recent study, Christoph Seeger and his colleagues showed a complete spectrum of mutations in HBV cccDNAs following Cas9 cleavage and repair by NHEJ. More than 90% of HBV DNAs were cleaved by Cas9. In addition, Cas9 cleavage and APOBEC-mediated cytosine deamination following treatment of infected cells with IFN-α were compared. The results indicated that the efficiency of editing HBV DNA after Cas9 cleavage was at least 15000 times higher than that of the latter (Seeger and Sohn, 2016). In conclusion, the researchers confirmed that the CRISPR/Cas9 system (Figure 1A) may be the best method for inactivation of HBV cccDNA function and it may provide a possible way to cure chronic hepatitis (Seeger and Sohn, 2016). This system has been used to target HBV DNA, including cccDNA in cell culture (Lin et al., 2014; Seeger and Sohn, 2014) and murine models (Lin et al., 2014).

For the CRISPR/Cas9 system, potential limitations need to be overcome, and the adverse effects should be carefully evaluated. The greatest concern is the ability to eradicate all viruses. The best result of HBV cleavage using CRISPR/Cas9 is a reduction of cccDNA by about 92% in cultured cells (Ramanan et al., 2015; Zhen et al., 2015). However, delivering the nucleases to every persistently infected cell in hepatic and extrahepatic viral reservoirs is the key to eradicating HBV, because HBV DNA can be also found in various other tissues outside the liver (Lin et al., 2015). It also has potential off-target effects (Fu et al., 2013). Although researchers have designed some treatment strategies of this type, these remain less efficient in practice. Another concern is how to select the proper target sites in the HBV genome among different viral genotypes. During the integration of linearized HBV, DNAs are cut by CRISPR/Cas9; the cleavage of integrated viral DNA can lead to indels in the host genomes, which may potentially disrupt the host gene function (Lin et al., 2015).

CONCLUSIONS AND PERSPECTIVES

In this review, recent progress in the study of cccDNA is summarized, but many problems have yet to be solved (Table 2); for example, whether chronic hepatitis B can be completely cured, that is, to eliminate cccDNA, or how to achieve a functional cure (control cccDNA expression leading past HBsAg loss) (Revill and Locarnini, 2016). At the same time, none of hepatocytes are damaged during the process of eliminating cccDNA.

The clinical application of antiviral drugs against HBV is still very limited. There are two types of antivirals for the treatment of chronic hepatitis B. IFN-α could modulate the host antiviral immune responses and nucleoside analogue could inhibit HBV DNA polymerase (Keeffe et al., 2008; Zoulim and Locarnini, 2009). However, these drugs cannot eliminate cccDNA. By using the HepDE19 system, researchers previously screened a small-molecule compound library and identified two disubstituted sulfonamide compounds that act as inhibitors of cccDNA formation by blocking RC-DNA deproteinization (Cai et al., 2012). So far, no drugs have been found directly targeting cccDNA, although some pro-

Table 2. Comparison of four strategies against HBV cccDNA

Strategies	References	Advantages	Limitations	Future directions
Host DNA damage response	Pommier et al., 2014; Königer et al., 2014; Yang et al., 2014; Cui et al., 2015; Qi et al., 2016; Oqi and Lehmann, 2006; Oqi et al., 2010; Ran et al. 2017; Schreiner and Nassal, 2017.	The present experimental evidence shows many members of the host DNA damage response, such as TDP2, DNA polymerase, especially DNA polymerase κ, and the host DNA ligases are involved in the conversion of RC-DNA to cccDNA.	Interference with DNA repair response may cause severe genetic instability of host hepatocytes.	The study of how the host DNA damage response is involved in the conversion of RC-DNA to cccDNA is just at its beginning. Through knockout of a defined DNA repair gene, an individual host factor in the DNA repair pathway can be investigated for its involvement in cccDNA formation.
Epigenetic regulation of cccDNA	Belloni et al., 2009; Guo et al., 2014; Rivière et al., 2015; Decorsière et al., 2016; Murphy et al., 2016; Niu et al., 2017; Schubeler, 2015; Vivekanandan et al., 2010; Hong et al., 2017; Palumbo et al., 2015.	HBx is required for cccDNA transcriptional activity; thus, it represents an attractive anti-cccDNA target. DNMTs could be utilized to inhibit cccDNA transcription. IL6 has a repressive effect on HBV replication.	The mechanism of HBx-mediated cccDNA transcription activation is to a large extent unclear. cccDNA functional inactivation, instead of elimination, may be only a transient solution. The role of DNMTs in HBV DNA methylation has not been clearly identified.	Epigenetic regulation of cccDNA transcription activity should be addressed in detail, including post-translational modifications of histones, nucleosome spacing, non-coding RNAs, etc.
Immune-mediated degradation	Lucifora et al., 2014; Luo et al., 2016; Kitamura et al., 2013.	High doses of IFN-α or LTβR-agonists were able to trigger noncytolytic degradation of cccDNA. This underscores the value of activating the innate immune response.	A fraction of the cccDNA pool is refractory to further reduction.	Studies, such as of the Toll-like receptor 7 agonist GS-9620, are indicative of the importance of reactivating host innate response.
Genome-editing nucleases	Seeger and Sohn, 2016; Lin et al., 2014; Ramanan et al., 2015; Zhen et al., 2015; Lin et al., 2015; Fu et al., 2013.	The application of artificial endonucleases has promoted cccDNA-related studies. More than 90% of HBV DNAs were cleaved by Cas9. The CRISPR/Cas9 system has been used to target HBV DNA, including cccDNA in cell culture and in the murine model.	It is so far unconfirmed that the nuclease has been delivered to each persistent infected hepatocyte. The potential off-target effects may have an adverse effect on liver cell function, especially in the presence of long-acting effector enzymes. The CRISPR/Cas9 system can lead to indels in the host genomes. Finally, excessive amounts of pre-existing RC-DNA may supplement the cccDNA source.	How nucleases enter into all HBV-infected hepatocytes to attack cccDNA, and how to avoid off-target effects of CRISPR/Cas9 etc. remain major challenges.

gress has been made towards developing small molecule anti-HBV compounds to interfere with other steps of HBV replication. HBV nucleocapsid assembly is considered as a potential target. Prototypes of small molecule modulators of HBV nucleocapsid assembly have been developed and partly tested through clinical phase I (Yang and Lu, 2017). Cyclosporin derivatives inhibit HBV entry with no decrease in NTCP transporter activity, so bile acid transport would not be influenced. These anti-HBV molecules may be potential candidates for developing novel drugs with fewer adverse effects in the near future (Shimura et al., 2017).

The underlying mechanism of cccDNA formation is not yet well understood, except that cccDNA starts with an RC-DNA molecule as a precursor prior to a series of biochemical steps. In the conversion of RC-DNA to cccDNA, many details such as the removal of P protein, the capped RNA primer and finally formation of closed loops are not yet clear. What we can be sure of is that HBV could not perform the multiple steps of changing RC-DNA to cccDNA without exploiting host nucleic acid enzymes that are derived from the DNA repair system. However, the host DNA repair system is a complex process and how it interferes with HBV or the formation of cccDNA has not been studied thoroughly. Understanding the molecular mechanism of the conversion of RC-DNA into cccDNA is critical, and may aid the development of strategies to cure chronic hepatitis B, and the host DNA repair system represents a direction that can be dug deep. cccDNA transcriptional activity is positively affected by HBx, which is suspected to be an oncogene. HBV expression influences DNA repair owing to HBx, which is believed to interact with DNA repair proteins (Guo et al., 2014; Murphy et al., 2016). So, HBx connects HBV to the host DNA damage response and becomes an attractive target. Two studies in HepaRG cells demonstrated that HBV could suppress innate responses, possibly via its P protein (Liu et al., 2015; Luangsay et al., 2015). However, other studies were suggestive that HBV is a stealth virus that simply goes undetected and, hence, it possesses the potential to escape immune system attacks (Niu et al., 2017). A fraction of the cccDNA pool seems to be refractory to further reduction from the results of recent studies (Lucifora et al., 2014; Luo et al., 2016; Kitamura et al., 2013). Anyway, for the consideration of re-waking host immune responses to eliminate cccDNA, there are still many difficulties to be overcome. At present, the unambiguous detection of HBV cccDNA is still difficult. The results of different methods for quantifying cccDNA are widely divergent in the same system. For example, Southern blotting and cccDNA-specific PCR have limitations (Nassal, 2015). The results of two methods are in many cases inconsistent and we don't know which of the results is believable. The main issue is how to distinguish cccDNA from sequence-identical non-cccDNA forms (Schreiner and Nassal, 2017). This situation has severely limited the assessment of cccDNA. Hence, it is urgently necessary to develop an internationally recognized standard test method.

In conclusion, the development of more effective, better-tolerated and affordable antiviral treatment is crucial for controlling the progression of hepatitis, cirrhosis and hepatocellular carcinoma. To achieve the ultimate cure of a true chronic hepatitis B, further basic studies and a more detailed understanding of cccDNA formation mechanisms are still needed.

ACKNOWLEDGMENTS

This study was supported by the Key Project of Hubei Province Natural Science Foundation (2014CFA075), the National Natural Science Foundation of China (31400153) and the Applied Basic Research Program (2015060101010033), Wuhan, China.

COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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