Review

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Attacking hepatitis B virus cccDNA – The holy grail to hepatitis B cure

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Summary

HBV deposits a covalently closed circular DNA form, called cccDNA, in the nucleus of infected cells. As the central transcription template, the cccDNA minichromosome is a key intermediate in the HBV life cycle. Its location in the nucleus makes cccDNA a difficult target for antivirals and immune response, and therefore it is responsible for chronicity of HBV infection. While little is known about the mechanisms involved in cccDNA formation, current research is accumulating data on the mechanisms regulating transcription from cccDNA, and the first potential targeting approaches have been reported. This review will summarize our knowledge about cccDNA biology and the latest advances in cccDNA targeting strategies in order to finally achieve an HBV cure.

Keywords: Hepatitis B virus; cccDNA; Liver.

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Introduction

Hepatitis B virus (HBV) has optimized its life cycle for long-term persistence in the liver. To achieve this, the virus establishes a plasmid-like covalently closed circular DNA (cccDNA) form. HBV cccDNA serves as a template for the transcription of all HBV RNAs and the production of progeny virus. HBV cccDNA resides in the nucleus of infected cells as an episomal (i.e. non-integrated) DNA associated with histones. It drives production of viral antigens such as HBeAg and HBsAg, and progeny virions. In contrast to cccDNA, the DNA contained in infectious virions is only partially double stranded, forming a relaxed circular DNA (rcDNA) that results from reverse transcription of a pregenomic RNA.

Available antivirals cannot cure HBV because they target reverse transcription as a late step in the life cycle and do not affect the cccDNA transcription template. Nucleos(t)ide analogues such as lamivudine, entecavir or tenofovir can efficiently control progeny release and thus viremia, but neither cccDNA persistence nor HBsAg or HBeAg expression and secretion. Similarly, neither entry inhibitors nor capsid assembly modifiers as novel antivirals under clinical development, target HBV cccDNA (see "New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus", Durantel and Zoulim [71]). Therefore, long-term treatment is required, which is expensive, challenges patient adherence and may lead to concomitant resistance [1]. Interferon (IFN)-α, which is also licensed for HBV therapy and has direct antiviral as well as immune modulatory effects, may result in HBsAg loss and even virus clearance in a proportion of patients, but dosing is limited by severe side effects [2]. Therefore, efficient removal of cccDNA from infected hepatocytes is regarded as the "holy grail" in achieving HBV cure. This review will summarize the different experimental models and detection assays available for the study of cccDNA, our current knowledge of cccDNA biology as well as potential therapeutic approaches to target cccDNA.

Experimental models to study the cccDNA fate

Historically, *in vitro* and *in vivo* models to study the fate of HBV cccDNA have been rather limited (see "Experimental *in vitro* and *in vivo* models for the study of human Hepatitis B virus infection", Allweiss and Dandri [70]). Only three cell culture models, primary tupaia, or human hepatocytes (PHH) and differentiated HepaRG (dHepaRG), have been used to facilitate HBV infection *in vitro* and thus, replication of HBV from its natural transcription template, the cccDNA. These models are very demanding and only used by specialized laboratories. The recent identification of the Na^+-taurocholate co-transporting peptide (NTCP) as a crucial HBV receptor [3], has allowed

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Abbreviations: HBV, Hepatitis B virus; rcDNA, relaxed circular DNA; PHH, primary human hepatocyte; dHepaRG, differentiated HepaRG; NTCP, Na^+-taurocholate cotransporting peptide; HuHEP, humanized liver-chimeric; RCA, Rolling circle amplification; pf-rcDNA, protein-free rcDNA; TDP, tyrosyl-DNA-phosphodiesterase; DSS, disubstituted sulfonamides; IFN-α, interferon alfa; TNF, tumor necrosis factor; LTβR, lymphotoxin beta receptor; A3A, APOBEC3A; A3B, APOBEC3B; IFNγ, interferon gamma; TALENs, TAL effector nucleases.

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Table 1. cccDNA levels and estimated time of maintenance of HBV infection in different *in vitro* and *in vivo* models.

	% of infected cells	cccDNA levels (average copies/nucleus)	Maintenance of infection
Primary human hepatocytes	20-100%	1 to 2	2-3 weeks [58]
dHepaRG	5-20%	0.2 to 0.5	>6 months [40]
HepG2-NTCP	50-100%	1 to 5	10-15 days [4]
HuHep mice	100%	1 to 2	>4 months [13]

Key point

The HBV cccDNA is a key intermediate in the virus life cycle that persists as an episome in the nucleus of infected cells.

Key point

Specific quantification of the HBV cccDNA remains a challenge, and there is a high need for consensus protocols.

the establishment of new cell lines that support HBV infection and the study of cccDNA *in vitro*.

While short-term experiments have been enabled by an improved HBV infection rate (and consequently higher levels of cccDNA) in NTCP-overexpressing HuH7, HepaRG and in particular HepG2 cells [3,4], experiments to study the fate and regulation of cccDNA over time remain challenging. Maintenance of infection for more than a month is only possible in HepaRG cells in culture, and is complicated further by their ability to differentiate into two different cell types (biliary like and hepatocyte like cells) and that they only establish low levels of cccDNA (see Table 1). Other cell lines continue to divide, resulting in loss of cccDNA (see below) adding further technical challenge to the research. Furthermore, it is important to note that despite recent improvements, *in vitro* HBV infections are still rather inefficient, requiring a huge excess of virus (100–10,000 virions/cell depending on the viral inoculum, the cell type and infection protocol used) and polyethylene glycol to facilitate virus fusion. Moreover, there is no evidence of efficient HBV spread in cell culture – unlike for other viruses.

In vivo models are limited by the strict species specificity of HBV, which only infects human and humanoid primates. Studies using chimpanzees have made a large contribution to our understanding of the virus-host interaction [5] but have recently been banned [6]. While chimpanzee hepatocytes support HBV replication [7–9], monkeys such as macaques or baboons, frequently used for research, cannot be infected by HBV due to differences in the NTCP protein sequence [10]. Macaques expressing the human NTCP will, however, constitute a very promising experimental model. Mouse hepatocytes grafted with NTCP support HBV entry but do not allow cccDNA establishment [11], probably because they lack key factors involved in nuclear transport of rcDNA or rcDNA to cccDNA conversion. Humanized liver-chimeric (HuHEP) mice allow long-term study of cccDNA [13] but are immune deficient and due to technical and cost related issues, cannot not be used for large-scale experiments. Understanding cccDNA biology, regulation and fate would therefore greatly benefit from further improvements of the HBV infection models.

The challenging detection of HBV cccDNA

With low copy numbers of cccDNA *in vitro* (Table 1) and *in vivo*, detection of cccDNA is a huge challenge for the HBV research field. Mean values from 0.01 to 1 copy per cell in livers of HBV-infected patients [14] and HuHEP mice [13] have been reported. This means that only a portion of the cells were actually infected by HBV, and methods to detect cccDNA on a per cell level are lacking. Moreover, true discrimination of cccDNA from the almost identical viral linear DNA or rcDNA increases the challenge, as these exist in vast excess within virions and viral capsids in the cytoplasm of infected cells. While Southern blot analyses can be used to distinguish between relaxed rcDNA, linear and supercoiled cccDNA, the sensitivity of this technique is low. To increase sensitivity, cccDNA selective qPCR methods have been developed based on the use of primers (and probes) spanning the nick in rcDNA and hybridizing to its "gap region" (Fig. 1). With the amazing technical development in PCR-based methods over the past years [15], the sensitivity of cccDNA detection is no longer an issue. Specificity of the cccDNA PCR methods, however, remain a big concern and correct quantification of cccDNA is hampered by false positive

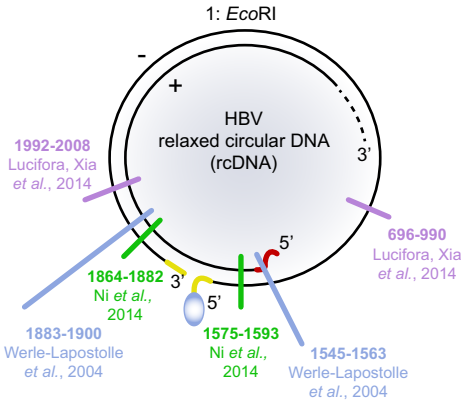

Fig. 1. cccDNA qPCR primers spanning the "nick region" of the HBV relaxed circular DNA (rcDNA). Examples of qPCR primers designed to selectively amplify cccDNA over rcDNA. Primers in purple are used with DNA intercalating reagents; primers in blue and green are used in combination with probe detection.