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Attacking Hepatitis B Virus cccDNA - the Holy Grail to Hepatitis B Cure

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

key words: hepatitis B virus, cccDNA, liver

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Abstract

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HBV deposits a covalently closed circular DNA form, called cccDNA, in the nucleus of infected cells. As the central transcription template, the cccDNA mini-chromosome is a key intermediate in the HBV life cycle. Being hard to target in the nucleus by antivirals, cccDNA is responsible for chronicity of HBV infection. While little is known about the mechanisms involved in cccDNA formation, data are currently accumulating on control mechanisms involved in regulating transcription from cccDNA, and the first potential targeting approaches have already been reported. This review will summarize our knowledge about cccDNA biology and latest advances in strategies with the potential to target cccDNA and to finally achieve an HBV cure.

Introduction

1
2 Hepatitis B virus (HBV) has optimized its life cycle for long-term persistence in the liver. To
3 achieve this, the virus establishes a plasmid-like covalently closed circular DNA form, the so-
4 called cccDNA. HBV cccDNA serves as template for transcription of all HBV RNAs and
5 production of progeny virus. HBV cccDNA resides in the nucleus of infected cells as an
6 episomal (i.e. non-integrated) DNA associated to histones. It drives production of viral
7 antigens such as HBeAg and HBsAg, and progeny virions. In contrast to cccDNA, the DNA
8 contained in infectious virions is an only partially double stranded, relaxed circular DNA
9 (rcDNA) that results from reverse transcription of a pregenomic RNA.

10 Available antivirals cannot cure hepatitis B because they target reverse transcription as a
11 late step in the HBV life cycle and do not affect the cccDNA transcription template.
12 Nucleos(t)ide analogues such as lamivudine, entecavir or tenofovir can efficiently control
13 progeny release and thus viremia, but neither cccDNA persistence nor HBsAg or HBeAg
14 expression and secretion. Similarly, neither entry inhibitors nor capsid assembly modifiers as
15 novel antivirals under clinical development target HBV cccDNA (see *"New antiviral targets
16 for novel treatment concepts for HBV"*, Durantel and Zoulim). Therefore, long-term
17 treatment is required, which is expensive, challenges patient adherence and may lead to
18 concomitant resistance [1]. Interferon (IFN)- α , which is also licensed for hepatitis B therapy
19 and has direct antiviral as well as immune modulatory effects, may result in HBsAg loss and
20 even virus clearance in a proportion of patients, but dosing is limited by severe side effects
21 [2]. Therefore, efficient removal of cccDNA from infected hepatocytes is regarded as the
22 "holy grail" to achieve HBV cure. This review will summarize the different experimental
23 models and detection assays available to study cccDNA, our current knowledge on cccDNA
24 biology as well as potential therapeutic approaches to attack cccDNA.
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Experimental models to study the cccDNA fate

51 For a long time, *in vitro* and *in vivo* models to study the fate of HBV cccDNA were rather
52 limited (see *Experimental models (cell culture and animals) for studies of human HBV
53 pathobiology and new treatment concepts*, M Dandri). Only primary tupaia or human
54 hepatocytes (PHH) and differentiated HepaRG (dHepaRG) were used allowing HBV infection
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1 and thus replication of HBV from its natural transcription template, the cccDNA. These
2 models, however, are very demanding. The recent identification of the Na⁺-taurocholate
3 cotransporting peptide (NTCP) as a crucial HBV receptor [3] allowed establishing new cell
4 lines that support HBV infection and the study of cccDNA *in vitro*.
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7 While short-term experiments were enabled by an improved HBV infection rate (and
8 consequently higher levels of cccDNA) in NTCP-overexpressing HuH7, HepaRG and in
9 particular HepG2 cells [3, 4], experiments to study the fate and regulation of cccDNA over
10 time remain challenging. Only HepaRG cells can be kept infected in culture for more than a
11 month, but they differentiate into two different cell types (biliary like and hepatocyte like
12 cells) and establish only low levels of cccDNA (see **Table 1**). Other cell lines keep dividing and
13 cell division results in loss of cccDNA (see below). This technically challenges our research. It
14 is furthermore important to note that despite recent improvements, *in vitro* HBV infections
15 are still rather inefficient, requiring a huge excess of virus (100-10,000 virions/cell depending
16 on the virus inoculum, the cell type and infection protocol used) and polyethylene glycol to
17 facilitate virus fusion. Moreover, there is no evidence of HBV spread in cell culture – unlike
18 for other viruses.
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21 *In vivo* models are limited by the strict species specificity of HBV that only infects human and
22 humanoid primates. Studies using chimpanzees largely contributed to our understanding of
23 the virus-host interaction [5] but have since been banned [6]. While chimpanzee
24 hepatocytes support HBV replication [7-9], monkeys such as macaques or baboons
25 frequently used for research cannot be infected by HBV due to differences in the NTCP
26 protein sequence [10]. Macaques expressing the human NTCP will, however, constitute a
27 very promising experimental model. Mouse hepatocytes grafted with NTCP support HBV
28 entry but do not allow cccDNA establishment [11], probably because they lack key factors
29 involved in nuclear transport of rcDNA or rcDNA to cccDNA conversion. Humanized liver-
30 chimeric (HuHEP) mice allow long-term study of cccDNA [13] but are immune deficient and
31 due to technical and cost issues cannot not be used for large-scale experiments.
32 Understanding cccDNA biology, regulation and fate would thus greatly benefit from further
33 improvements of the HBV infection models.
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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 copies per cell in livers of HBV-infected patients [14] or HuHEP mice [13] have been reported. This means that only a portion of the cells were actually infected by HBV, but methods to detect cccDNA on a per-cell level are lacking. Moreover, true discrimination of cccDNA from almost identical viral linear DNA or rcDNA increases the challenge, because these exist in vast excess in virions and in 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 rather low. To increase sensitivity, cccDNA selective qPCR methods have been developed that are based on the use of primers (and probes) spanning the nick in rcDNA and hybridizing in its “gap region” (**Figure 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, remains a big concern and correct quantification of cccDNA is hampered by false positive detection of rcDNA HBV genomes that are present in vast excess in infected cells. Rolling circle amplification (RCA) coupled or not with nested PCR has been described to increase specificity of cccDNA detection [16-18] but has not been widely accepted. Extraction of nuclear DNA only [19], plasmid safe DNase that digests linear viral DNA, but not rcDNA [20] and T5 exonuclease that degrades rcDNA but should leave cccDNA molecules intact (Xia et al, Hepatitis B Virus: Methods and Protocols, in press) can also be used to increase specificity. Of note, these exonucleases have different efficacy depending on the type and amount of “contaminating” DNA. So far, laboratories apply different protocols and a consensus or standard has yet to be reached. This, however, should be of high priority given that also the protocol of DNA extraction may influence cccDNA detection by generating nicks in cccDNA molecules that may render them susceptible to exonuclease treatment, may prevent RCA detection and may change the running behavior in gels before Southern blotting.

Establishment of HBV cccDNA in the nucleus

Upon infection, cccDNA is formed as a plasmid-like episome in the host cell nucleus from the

1 rcDNA genome in incoming virions that is linked to the viral polymerase protein.
2 Alternatively, cccDNA may be established from capsid rcDNA that can be reimported into the
3 nucleus after it has been newly formed in infected cells. cccDNA was recently detected in a
4 mouse hepatocyte cell line immortalized by stable expression of the human transforming
5 growth factor alpha [12] supporting the fact that human host factors are essential for
6 cccDNA formation and more precisely for destabilization of mature nucleocapsids. Nuclear
7 localization signals within the arginine-rich C-terminal domain of the HBV core protein [21]
8 trigger transport of the rcDNA-containing HBV capsid to the nucleus where the capsid
9 attaches to nucleoporin 153 and is disintegrated [22]. Upon translocation into the nucleus,
10 the conversion of the rcDNA genome into cccDNA takes place via a multi-step process that
11 remains largely obscure although it has been linked to DNA repair mechanisms [23].
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21 Firstly, the rcDNA needs to be detached from the HBV polymerase and its RNA primer that
22 are remnants from reverse transcription that took place within the viral capsid. Hereby, a
23 protein-free rcDNA form (pf-rcDNA, equivalent to DP-rcDNA for deproteinized rcDNA) is
24 generated. Pf-rcDNA may persist in the nucleus before conversion into cccDNA, and has
25 even been observed in the cytoplasm [24-26]. Secondly, the incompleteness of the rcDNA (+)
26 strand (the “gap”) and the terminal redundancy in its (-) strand (the “nick”) must be repaired
27 (**Figure 2**). It has recently been demonstrated that tyrosyl-DNA-phosphodiesterase (TDP) 2
28 can specifically cleave the Tyr-DNA bond to release the polymerase protein from HBV rcDNA
29 [27]. The fact that TDP2 knockout HepG2-NTCP cells engineered by a CRISPR/Cas9-based
30 approach still proved susceptible to HBV infection [28] indicated that alternative pathway(s)
31 exist involving nucleolytic cleavage by (a) still elusive endonuclease(s). Comparison of HBV
32 and duck hepatitis B virus cccDNA formation revealed an unexpected contribution of the
33 virus itself to cccDNA formation. DHBV but not HBV rcDNA converted efficiently into cccDNA
34 in the same cells [20]. Linear HBV DNA, a probably accidental by-product of reverse
35 transcription, cannot be transcribed per se but may form cccDNA-like molecules by non-
36 homologous end joining. Most of these, however, will be functionally defective [23].
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57 **Structure and regulation of the HBV cccDNA**

58 Once formed, cccDNA persists as an episome in the nucleus of infected cells and viral mRNAs
59 are transcribed by the cellular RNA polymerase II. The cccDNA molecule is organized into a
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1 chromatin-like structure that displays the typical beads-on-a-string arrangement in electron
2 microscopy [29, 30]. Attached proteins are host histone but also non-histone proteins [31].
3 During the last years major efforts have been made to demonstrate that the transcriptional
4 activity of cccDNA is subject to the “histone code” both *in vitro* and *in vivo* [19, 32, 33]. Using
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ChIP approaches, the recruitment of H3 and H4 histones, as well as that of several cellular transcription factors (including CREB, ATF, STAT1/2) and histone modifying enzymes (such as CBP, p300, PCAF/GCN5, HDAC1, hSirt1, PRMT1) has been demonstrated and was associated with modulation of cccDNA transcriptional activity [19, 32, 34]. Using a chromosome immunoprecipitation approach followed by next generation sequencing, the first genome-wide map of histone posttranslational modifications in cccDNA-associated chromatin was established [35]. This revealed that HBV chromatin shares some basic features with cellular chromatin, in particular marks linked to active transcription, but lacks significant levels of posttranslational histone modifications associated with transcriptional repression, which, in cellular chromatin, are indispensable for transcriptional control [35]. When applied to *de novo* infected HepG2-NTCP cells or primary human hepatocytes or to HBV-infected liver tissue the analysis revealed general features of cccDNA chromatin organization as well as features that are unique to the respective source of cccDNA [35]. This emphasizes the importance to repeat experiments and to confirm results using different infection models.

Despite being named a “minichromosome”, HBV cccDNA unlike other chromosomes does neither contain an origin of replication nor possess a nuclear retaining signal. It thus cannot replicate by itself in infected hepatocytes and should theoretically be excluded from the nucleus after cell division. cccDNA persistence therefore relies on its stability in the nucleus and on its replenishment via reimport of rcDNA from cytoplasmic capsids after cell division. The half-life of HBV cccDNA has not been exactly defined and varies with inflammatory activity resulting in hepatocyte turnover. Rebound of HBV replication upon withdrawal from nucleos(t)ide analogue treatment [36] and traces of HBV-DNA remaining detectable years after clinical recovery from acute hepatitis [37], however, indicate that HBV cccDNA is very long-lived in the human liver and can persist for decades. *In vitro*, cccDNA also appears to be very stable [38, 39] independently of its transcriptional activity as demonstrated in HepaRG cells infected with an HBx-deficient HBV unable to transcribe mRNA [40]. Since it is not self-maintained, cccDNA should theoretically be lost by cell division e.g. in case of liver regeneration. However, cccDNA may survive through mitosis even though it has been

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observed that induction of cell division results in a dramatic decrease of cccDNA [41-45]. This may be due to a “refill” via reimport of rcDNA from newly formed viral capsids into the nucleus.

A combination of *in vitro* approaches, including electromobility shift and DNA protection assays, has shown that a number of transcription factors bind to HBV regulatory elements [46]. Among them, HNF1 α and HNF4 α are the crucial nuclear factors for HBV transcription needed at sufficient levels [47, 48]. Viral proteins are also very important in cccDNA structure and control. In particular, the non-structural protein HBx is associated with cccDNA [32] and essential to initiate and maintain transcription from cccDNA [40]. Indeed, using dHepaRG cells in which HBx is expressed in a tetracyclin-regulated fashion and infecting them with an HBx-deficient HBV mutant, we showed that transcription of viral RNA exactly followed the switching on and off of HBx expression [40]. Hereby it appeared that HBV transcription is repressed in the absence of HBx rather than HBx functioning as a typical viral transactivator. This repression is characterized by a more compact structure of cccDNA [49] and associated with repressive epigenetic marks [32, 49] on cccDNA that were absent in the presence of HBx [35]. Interestingly, it was shown that HBx supports HBV gene expression by a conserved mechanism that acts specifically on episomal DNA templates and requires HBx binding to the cellular DDB1 E3 ubiquitin ligase [50]. Altogether these data suggest that HBx may block cellular factor(s) inducing mechanisms that inhibit transcription from cccDNA such as SETDB1 [49], Spindlin1 [51] as well as essential other factors that were reported at the recent international HBV meeting and will be published soon. In addition to HBx, the HBV core protein (HBc) binds to cccDNA resulting in a rearrangement of the nucleosomal spacing of the HBV nucleoprotein complexes [31]. Whether HBc also regulates transcription from cccDNA is still debated. On one hand, it was shown that HBc alters the cccDNA methylation profile and therefore maintains a permissive epigenetic state in a critical region of the HBV cccDNA [52]. On the other hand, a decreased recruitment of HBc on HBVx- cccDNA was recently described but did not account for the failure in cccDNA transcription [49]. The origin of HBc attached to cccDNA (i.e. whether it stems from the incoming virus or from viral replication), the kinetic of its recruitment onto cccDNA and its stability once bound to cccDNA remain unresolved questions that would certainly help to understand its role on cccDNA regulation. Noteworthy, as HBc is important for cccDNA structure and/or control,

1 capsid assembly modifiers (CpAMs) that are currently in clinical development were
2 hypothesized to also have an effect on cccDNA [62]. This, however, needs to be proven.
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7 **Targeting HBV cccDNA to achieve HBV cure**

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10 Different options to target cccDNA to achieve affect HBV persistence are available: inhibition
11 of its formation, inhibition of its function or inducing its degradation (**Figure 3**).
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14 Two structurally related disubstituted sulfonamides (DSS) were shown to interfere with
15 rcDNA conversion into cccDNA [38]. Given the proposed contribution of the DNA-damage
16 response in this process, viral proteins or substances interfering with the DNA-damage
17 response should also interfere with rcDNA to cccDNA conversion. Considering the long half-
18 life of cccDNA, however, these compounds should only have an effect if given during the
19 establishment of HBV infection or during phases of high hepatocyte turnover.
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22 Once cccDNA is formed its transcription can be inhibited by cytokines. For instance, we
23 demonstrated that IL-6 inhibits HBV transcription from cccDNA by reducing the levels of the
24 essential transcription factors HNF1 α and HNF4 α through activation of the MAP kinase
25 pathway [53]. This results in a loss of HNF1 α and HNF4 α binding to the cccDNA, which is
26 accompanied by a redistribution of STAT3 binding from cccDNA to cellular IL-6 target genes
27 [48]. It has also been shown that cccDNA transcription can be silenced by the use of IFN- α or
28 small molecules targeting chromatin modifying enzymes [34, 54-56]. Genome-wide mapping
29 of histone modifications revealed that transcription and active modifications in HBV
30 chromatin can be reduced when innate immunity pathways are activated, or by a small
31 molecule epigenetic modifying agent [35]. This supports the idea that chromatin-based
32 regulation of cccDNA transcription could be of therapeutic use. A better understanding of
33 the function of HBx protein and its interaction with DDB1 in the E3 ubiquitin ligase complex
34 [57], that potentially results in degradation of host proteins able to control HBV, may allow
35 to identify new targets for antiviral drug discovery. Because these strategies that aim at
36 inhibiting transcription from cccDNA will likely only have a transient effect, they may help to
37 achieve at least a functional cure of HBV.
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57 Purging cccDNA from the nucleus of infected cells is a much more promising approach since
58 this will result in a sustained control of HBV or even in HBV cure. In chimpanzees, it has been
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shown that clearance of HBV replication, but also HBV cccDNA, occurs before full-blown cytotoxicity of T cells hits the liver in acute hepatitis B [5]. This indicates that the cccDNA pool can be affected by non-cytolytic mechanisms involving cytokines such as IFNs and tumor necrosis factor (TNF) [5]. Recently, we showed that induction of intrahepatic antiviral immune responses (through high doses of IFN- α or LT β R-agonists) is able to trigger non-cytolytic degradation of cccDNA from infected cells *in vitro* [58]. Hereby, activation of nuclear deaminases such as APOBEC3A (A3A) and APOBEC3B (A3B) results in cccDNA deamination and a significant reduction of cccDNA [58]. Although A3A or A3B-mediated deamination was essential, the exact pathways and enzymes involved in downstream degradation of HBV-DNA still need to be deciphered. They are likely contributing to base-excision or nucleotide-excision repair pathways. It should also be determined if cccDNA can be fully purged or if a portion might be refractory to the treatments. Next we examined whether the mechanism of A3A/B-mediated deamination might contribute to T cell-mediated loss of cccDNA. We found that T-cell derived cytokines IFN γ and TNF induce A3A, and to a minor extend also A3B in a synergistic fashion [59]. To study whether these and eventually also other T cell-derived cytokines contribute to cccDNA loss in a non-cytolytic fashion, we set up a transwell system, which allowed us to separate cytolytic and non-cytolytic T cell functions. Hereby, we took advantage of chimeric antigen receptor (S-CAR) grafted T cells [60, 61] that recognize HBsAg on the surface of infected cells but could confirm the findings when T cells carried the natural T cell receptor. While upon direct contact in co-culture T cells killed HBV infected cells, they were only able to release cytokines when separated by the transwell. cccDNA loss, however, was observed in both settings and clearly A3A/A3B dependent [59]. Thus, HBV-specific T cells were able to eliminate HBV in a cytolytic and in a non-cytolytic fashion. This shows that cccDNA degradation in a non-cytolytic fashion is possible and may support HBV cure approaches by therapeutic vaccination, T cell redirection or other immune cell activation strategies such as e.g. PD-1 or PD-ligand1 blockade (see “*New antiviral targets for novel treatment concepts for HBV*”, Durantel and Zoulim).

Finally, as an alternative to immune activation that may result in tissue damage on the long term if not successful in eliminating HBV, DNA cleaving enzymes, including homing endonucleases or meganucleases, zinc-finger nucleases, TAL effector nucleases (TALENs), and CRISPR-associated system 9 (Cas9) proteins, specifically targeted to the HBV cccDNA are

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currently being engineered [63-69]. Clinical development of these interesting strategies, however, is limited by potential off-target effects resulting in cellular DNA damage. In addition, efficient delivery strategies targeting all infected hepatocytes are lacking and therapeutic application would currently require a gene therapy approach.

Taken together, further basic research and more detailed molecular studies are needed to evaluate the translational potential of the novel antiviral strategies mentioned to finally achieve a true HBV cure.

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37 **Figure and table legends**

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

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

Figure 2: Schematic representation of rcDNA conversion to cccDNA. (i) Removal of viral polymerase linked to the 5' end of the (-) DNA strand and of one of the redundant sequences, (ii) removal of the RNA primer linked to the 5' end of the (+) strand, (iii) fill in and completion of the viral (+) DNA strand, and (iv) ligations of DNA extremities for both (+) and (-) strands. (v) Once formed, cccDNA is supercoiling and associating with proteins, such as histones, to form the so-called cccDNA mini-chromosome.

Figure 3: Targeting cccDNA to achieve HBV cure.

*EMA = epigenetic modifying agent; **HBP = HBx binding partner activation

	% 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⁾

Figure 1

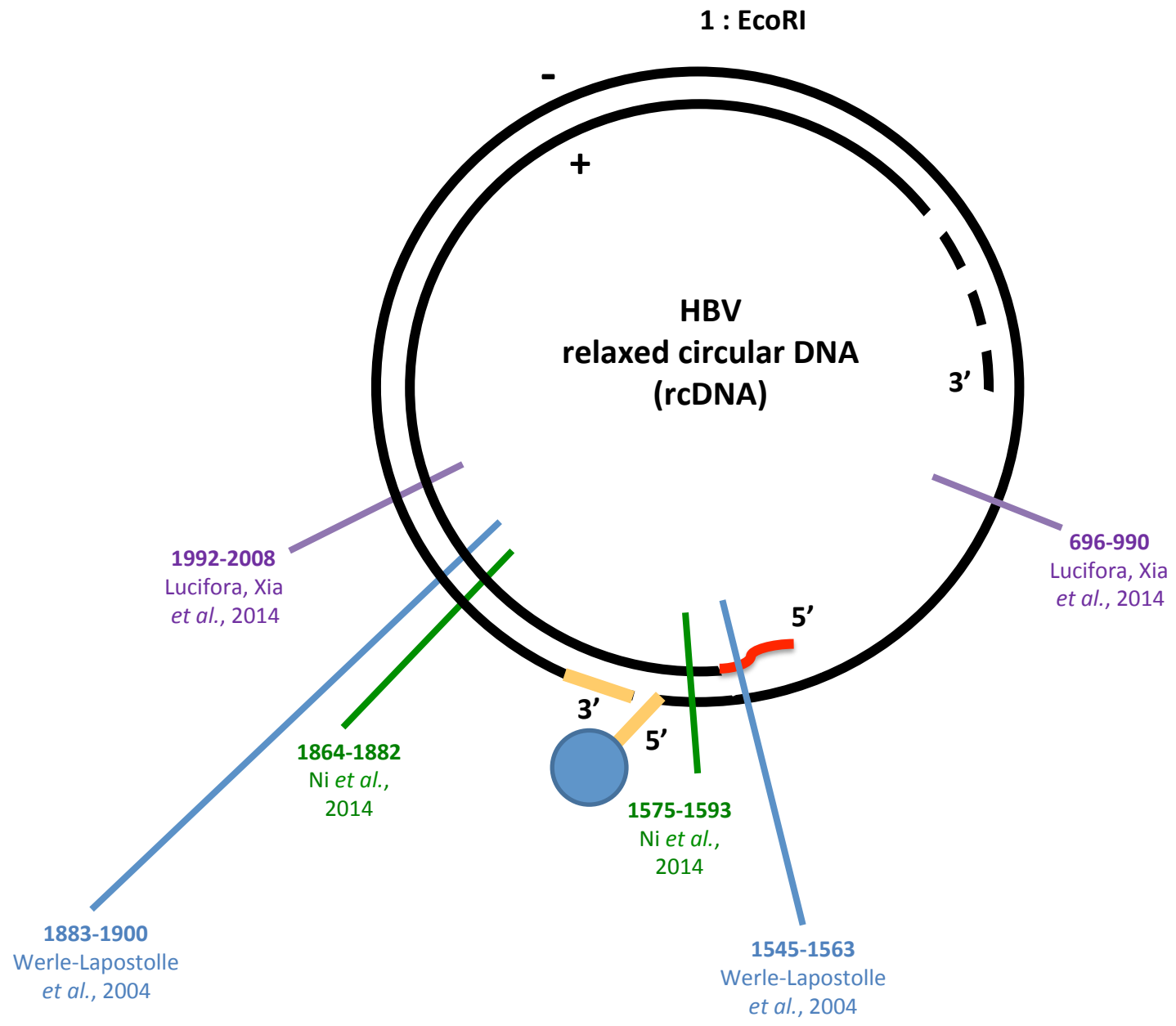


Figure 2

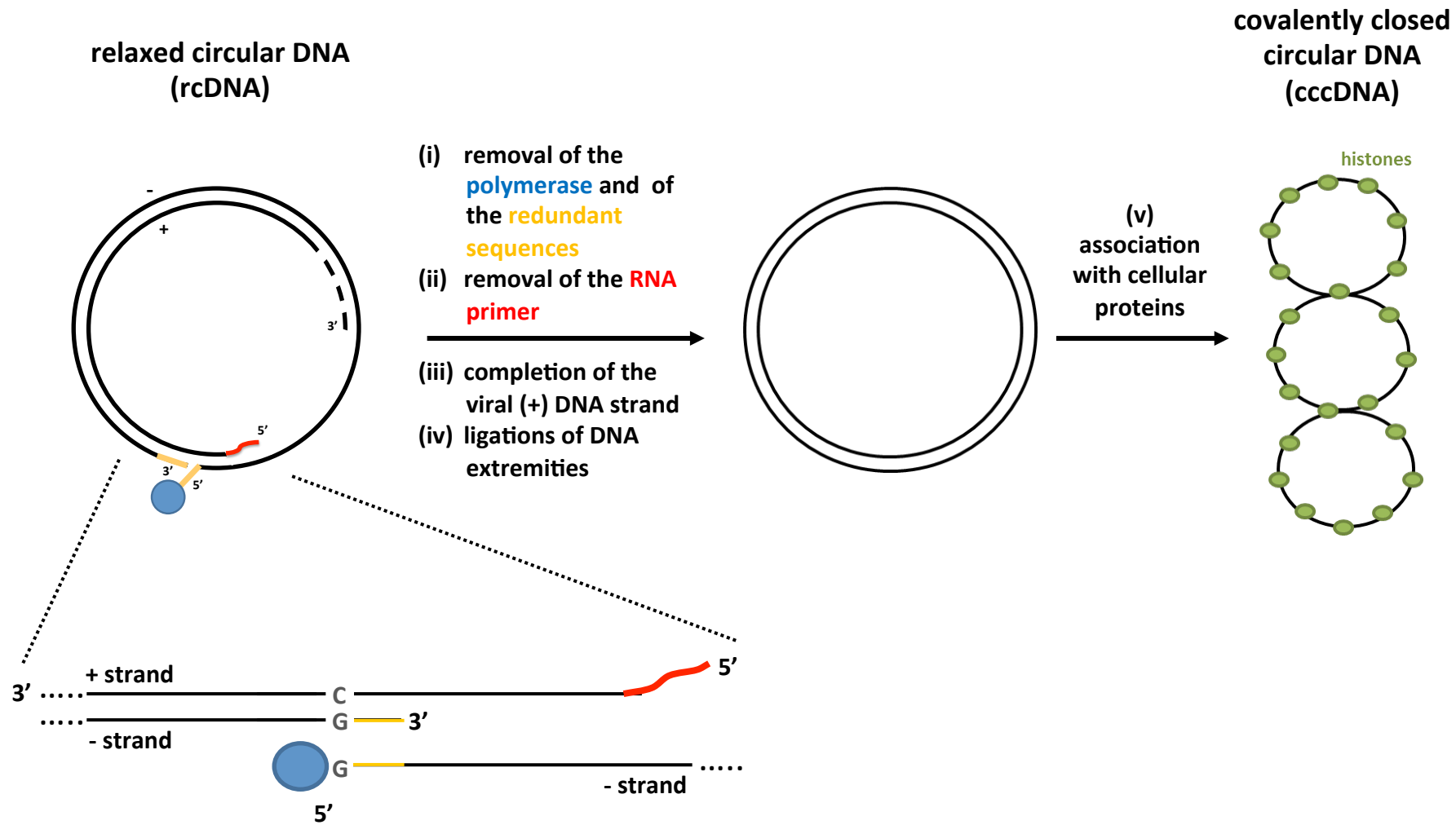


Figure 3

