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Review

Control of cccDNA function in hepatitis B virus infection☆

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The template of hepatitis B virus (HBV) transcription, the covalently closed circular DNA (cccDNA), plays a key role in the life cycle of the virus and permits the persistence of infection. Novel molecular techniques have opened new possibilities to investigate the organization and the activity of the cccDNA minichromosome *in vivo*, and recent advances have started to shed light on the complexity of the mechanisms controlling cccDNA function. Nuclear cccDNA accumulates in hepatocyte nuclei as a stable minichromosome organized by histone and non-histone viral and cellular proteins. Identification of the molecular mechanisms regulating cccDNA stability and its transcriptional activity at the RNA, DNA and epigenetic levels in the course of chronic hepatitis B (CH-B) infection may reveal new potential therapeutic targets for anti-HBV drugs and hence assist in the design of strategies aimed at silencing and eventually depleting the cccDNA reservoir.

***Keywords*: Hepatitis B virus; Covalently closed circular DNA; Chronic hepatitis B infection; cccDNA function**

Infection with hepatitis B virus (HBV) continues to be a major health problem with about 400 million people chronically infected worldwide who are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV is a member of the *Hepadnaviridae* family, that includes small enveloped DNA viruses infecting primates, rodents, and birds. One common characteristic of these viruses is their high species and cell-type specificity, as well as a unique genomic organization and replication mechanism.

1. cccDNA and HBV replication

Upon infection of the hepatocyte, the core nucleocapsid is released into the cytoplasm and the genomic DNA is transferred to the cell nucleus, where the relaxed circular (RC), partially double-stranded DNA is converted into a covalently closed circular DNA (cccDNA) molecule [2,3] (Fig. 1). Biochemically, the formation of cccDNA from RC DNA requires: (a) the removal of the viral polymerase covalently attached to the 5′ end of the negative DNA strand; (b) the removal from the 5′ end of the plus-strand DNA of the short RNA oligomer which is used to prime the

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Abbreviations: HBV, hepatitis B virus; CH-B, chronic hepatitis B; HCC, hepatocellular carcinoma; RC, relaxed circular; PF-RC DNA, protein free relaxed circular; cccDNA, covalently closed circular DNA; NHEJ, nonhomologous end joining; DLS, double-stranded linear (-DSL); pgRNA, pregenomic RNA; LS, viral large surface protein; C-hIP, chromatin immuno-precipitation.

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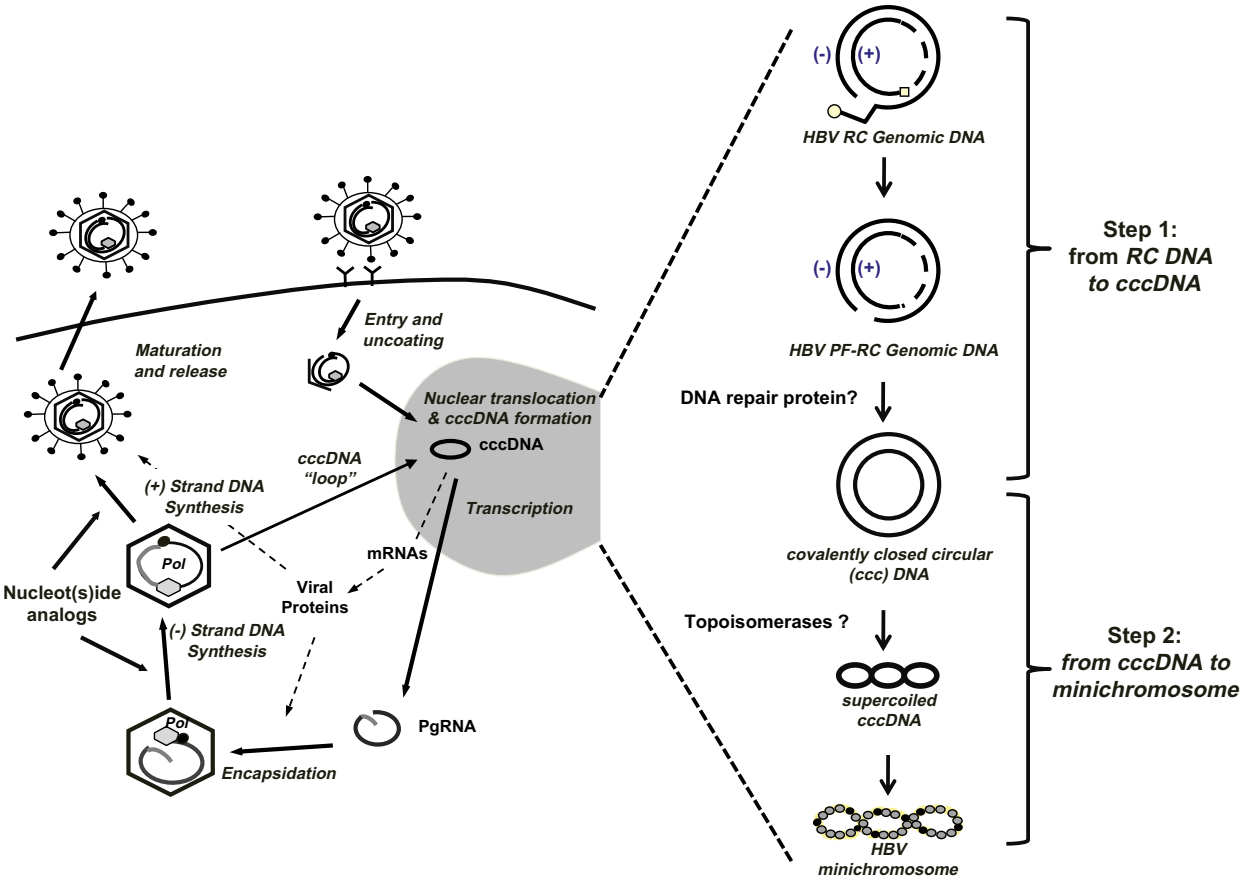


Fig. 1. HBV cccDNA in the viral life-cycle.

plus-strand DNA synthesis; (c) the removal of precisely one copy of the short terminal redundancy (“r”) from the minus-strand DNA; (d) the completion of the positive strand, which is variable in length in the RC DNA, by the cellular replicative machinery and (e) the ligation of the two viral DNA strands (reviewed in [4]). In hepatoma cell replication systems, the HBV cccDNA derives predominantly from RC DNA with a precise junction sequence and is accompanied, differently from DHBV replicating cells and human infected livers, by the accumulation of the “protein-free or PF-RC DNA” intermediates, from which the covalently attached viral reverse transcriptase (RT) protein has been removed [5]. PF-RC DNA, in contrast to RT-linked RC DNA contains almost exclusively, mature plus-strand DNA, suggesting that the RT protein is removed preferentially from RC DNA in mature core particles [5]. A small amount of cccDNA can also be generated from the so-called double-stranded linear (DSL) form of HBV-DNA [6] by intramolecular ligation of the linear DNA ends via nonhomologous end joining [NHEJ] [7]. However, this process of “illegitimate replication” leads to the formation of a cccDNA that harbors small deletions and/or insertions that preclude viral DNA replication.

Disguised as a stable non-integrated minichromosome, the cccDNA is not replicated by the host’s semi-conservative DNA synthesis machinery, but it utilizes the cellular transcriptional machinery to produce all viral RNAs necessary for protein production and viral replication, which takes place in the cytoplasm after reverse transcription of the pregenomic RNA (pgRNA) [8]. Of note, experiments performed using the duck model revealed that the major source of cccDNA in infected hepatocytes comes from newly synthesized nucleocapsids, which are not enveloped and secreted into the blood, but are transported into the nucleus to ensure accumulation, and later maintenance of the cccDNA pool [9,10]. Thus, these studies indicate that multiple rounds of infection are not needed to establish a stable cccDNA pool in infected cells. Evidence from animal models indicated that 1 to 50 cccDNA molecules accumulate per cell [3,11–13] and analysis of cccDNA distribution at the single cell level revealed that cccDNA amount varied significantly from cell to cell in the course of chronic infection in the duck model, where a fraction of cells was found to contain only one cccDNA copy [14]. However, the viral and host factors that control cccDNA formation and cccDNA pool size are yet poorly defined. A negative-feedback mechanism might

involve the viral large surface (LS) protein of DHBV suppressing cccDNA amplification [15,16]: when nuclear cccDNA levels reach 10–50 copies sufficient LS protein is made to shut off efficiently the cccDNA amplification pathway and redirect mature nucleocapsids (containing RC DNA) to envelopment and extracellular secretion.

2. Control of cccDNA pool in hepadnavirus infection

cccDNA is very stable in quiescent hepatocytes and the size of cccDNA pool, once established, may be reduced through different mechanisms that entail cell killing, dilution by cell proliferation and cell "cure" mediated by inflammatory cytokines. More recently, the possibility that the functionality of persisting cccDNA might be controlled by a reduction of its transcriptional activity has been proposed. Most of our knowledge on cccDNA pool size control in infected livers comes from transient and chronic hepatitis B infection in the woodchuck model [17]. It has to be emphasized, however, that differences may exist in both cccDNA dynamics and the efficiency and mechanisms of viral DNA integration into the host genome between HBV and WHV. In general, cccDNA can persist throughout the life span of the quiescent hepatocytes without affecting their viability. On the contrary, cell division may favour dilution/loss of cccDNA during mitosis, so that selection of cccDNA-free cells may occur when cells are forced to divide to compensate the loss of infected cells killed by the T cell response. In the acute phase hepadnavirus infection can spread to the entire hepatocyte population in the liver and, the uninfected hepatocytes present in the recovered liver have been shown to derive from previously infected hepatocytes [17,18]. During virus clearance antiviral cytokines are thought to block virus replication and the formation of new cccDNA. Clearance of infected hepatocytes from both replicative intermediates and cccDNA would occur through three modalities. In the first scenario, cccDNA is primarily eliminated by cytokines and cell death or cell proliferation do not contribute significantly [the "cure" model]. In the second, cytokines suppress cytoplasmic viral replication and new cccDNA formation but do not act on pre-existing cccDNA that is eliminated by both cell death and mitotic loss and would need a turnover of approximately 0.7 of the total liver mass [the "death and compensatory proliferation" model]. In the third, cccDNA survives hepatocytes mitosis, is distributed in a binomial fashion to the progeny hepatocytes and it is eliminated only by the elimination of infected cells, requiring an estimated turnover exceeding 2.5 times the total liver mass [the "cell death" model] (reviewed in [17]). An elegant computer modelling study based on the analysis of hepatocyte turnover during the resolution of untreated or entecavir-treated transiently infected woodchucks, indicated that cccDNA clearance requires, in addition to cell death, a combination of cytokine suppression and mitotic loss [19]. In chronically infected woodchuck livers, the identification of uninfected cell clones, which have lost cccDNA, but still contain "traces" of the infection in form of viral integrations has been explained as a cccDNA clearance without cell destruction [20,21]. Since liver cell death and regeneration usually occur in chronically infected woodchuck livers, an alternative hypothesis might be that surviving hepatocytes harbouring both viral integrations and cccDNA may divide, cccDNA would be lost through cell division while the integrated DNA would persist in daughter cells [20,21]. Thus, in chronic infection, killing of hepatocytes may be instrumental not only to eliminate infected cells but also to induce hepatocyte proliferation which, in turn, may favour cccDNA loss. On the other hand, studies have shown that very low levels of cccDNA can persist indefinitely, possibly explaining life-long immune responses to HBV despite clinical resolution of HBV infection [22,23].

As HBV polymerase inhibitors do not directly affect the cccDNA, a decrease in cccDNA levels is supposed to derive from the lack of sufficient recycling of viral nucleocapsids to the nucleus, due to the strong inhibition of viral DNA synthesis in the cytoplasm, and less incoming viruses from the blood. Following this scenario, cccDNA depletion will require many years of nucleos(t)ide drug administration and it is expected to lead to the selection of resistant variants [24]. Importantly, currently available nucleos(t)ide analogs do not prevent cccDNA formation when cells are exposed to a new round of viral infection [25–27], which in turn has important clinical implications in terms of treatment duration as persisting viremia during the antiviral treatment might be responsible for new rounds of infection. Simultaneous treatment with antiviral agents acting through different mechanisms and, possibly, limiting hepatocytes reinfection [28–31], as well as the development of novel therapeutic strategies aimed at eradicating cccDNA from the liver may be necessary to eventually achieve clearance of infection.

3. Structure of the cccDNA minichromosome

Nuclear hepadnaviral cccDNA molecules are organized into a chromatin-like structure as a viral minichromosome that displays the typical beads-on-a string arrangement at electron microscopy and reveals the nucleosomal packaging of transcriptionally inactive chromatin [3,32]. Organization of viral DNAs into a nucleosomal structure has been described for the polyoma viruses [33], simian virus 40 (SV40) [34], human (HPVs) [35] and bovine papilloma viruses (BPV) [36],

adenovirus [37], latent EBV [38] and Herpes simplex 1 [39] viruses. These viruses, with the possible exception of Herpes simplex, all produce covalently closed double-stranded DNAs (cccDNAs) that are found in the nuclei of infected cells. Two populations of Hepadnaviral minichromosomes, corresponding to a full or half complement of nucleosomes, can be detected possibly reflecting dynamic changes related to transcriptional regulation [3]. HBV minichromosome has been shown to consist of both histone and non-histone proteins [40]. Histone proteins H3 and H2B were the most prominent species but lower levels of histone proteins H4, H2A, and H1 were also detectable by immunoblotting on purified nuclear nucleocapsid complexes [40]. The virally encoded core protein is a structural component of the HBV minichromosome, binds preferentially to HBV double-stranded DNA and its binding results in a reduction of the nucleosomal spacing of the HBV nucleoprotein complexes by 10% from 200 bp to 180 bp [40]. A combination of *in vitro* approaches, including classical oligonucleotide gel retardation (EMSA) and DNAse protection assays, have shown that a number of transcription factors bind *in vitro* to the HBV enhancers 1 and 2 and regulate transcription driven by the same HBV regulatory elements [41] but their association with the viral minicromosome has not been proven *in vivo*. The chromatin immuno-precipitation (ChIP) method is a powerful technique that allows the identification *in vivo*, at a resolution of a few hundred base pairs, of the DNA binding sites of virtually any chromosome component [42,43]. The principle relies upon the fixation of protein–DNA and protein–protein interactions *in vivo* by the cross-linking agent formaldehyde. Chromatin is then disrupted by sonication, and the cross-linked protein of interest immuno-precipitated with specific antibody directed against histones, covalently modified (acetylated, methylated, SUMOlated etc.) histones, transcription factors, transcriptional co-

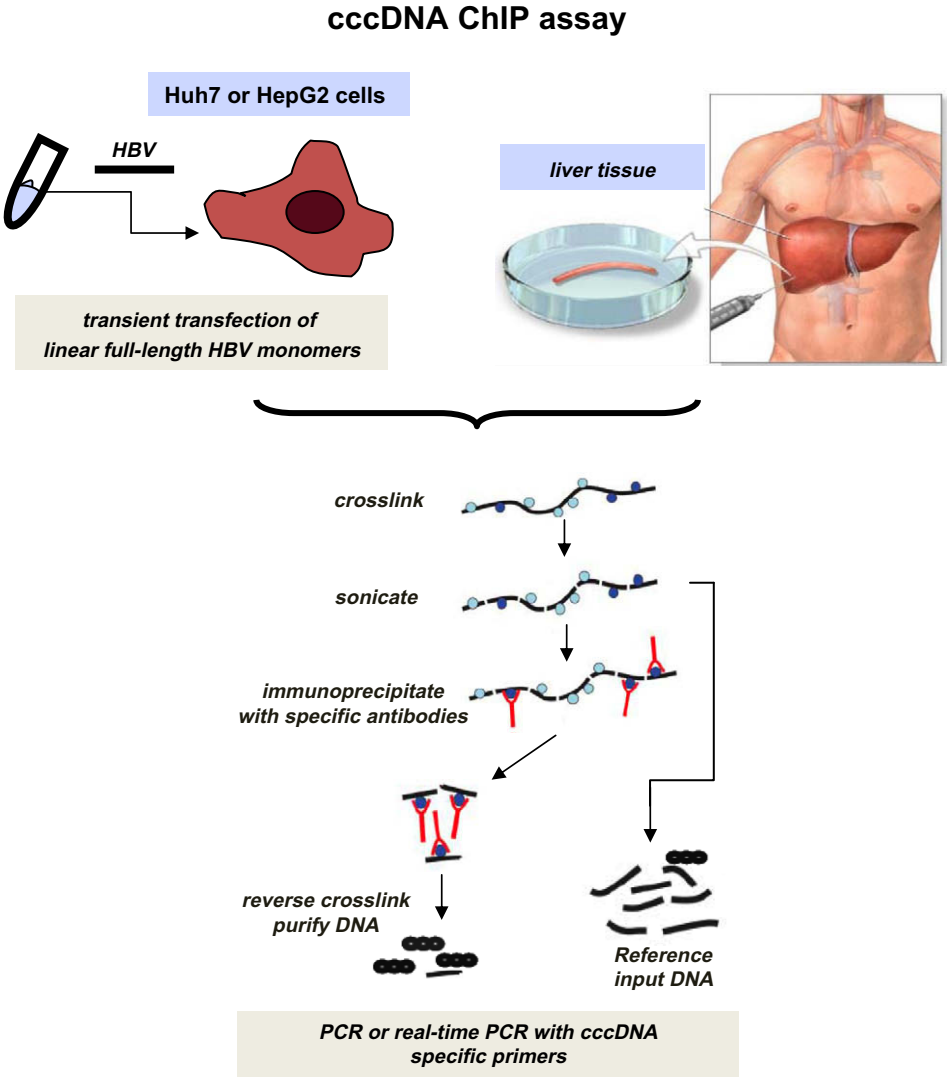


Fig. 2. The cccDNA chromatin immuno-precipitation assay.

activators and co-repressors or their post-translationally modified versions. After the cross-links are released by heating the immuno-precipitated DNA is used as a template in classic or real-time PCRs using oligonucleotides that amplify the region presumably bound by the protein of interest. ChIP-based protocols have evolved over time to work on as low as 1000 cultured cells as well as on a variety of surgical and biopsy tissue samples. We have recently developed a ChIP-based quantitative technique to study the recruitment *in vivo* of cellular and viral proteins onto the HBV minichromosome [44] (Fig. 2). The HBV cccDNA ChIP assay combines a cccDNA ChIP step with a sensitive and specific real-time PCR protocol for cccDNA quantification [45].

The assay has been first used to immunoprecipitate and analyse the cccDNA in a cellular model of HBV replication based upon the transient transfection of linear full-length HBV monomers into HuH7 and HepG2 hepatoma cells [46] and subsequently adapted to study the cccDNA in baculovirus-HBV-infected cells [47], hepatocytes derived from the livers of humanized chimera mice infected *in vivo* with HBV (Dandri and Belloni, unpublished observations) and liver biopsy samples from chronic hepatitis B patients [44]. Using the cccDNA-ChIP assay we and others have confirmed the recruitment *in vivo* of H3 and H4 histones [44,48] and of HBV core proteins on the cccDNA minichromosome [44]. Using the same approach several cellular transcription factors, including CREB, ATF, STAT1 and STAT2, and chromatin modifying enzymes have been shown to bind to the cccDNA in cells replicating HBV [49, Belloni, unpublished observations]. The demonstration that the cellular histone acetyltransferases CBP (a coactivator of both CREB and ATF transcription factors), p300 and PCAF/GCN5 and the histone deacetylases HDAC1 and hSirtl are recruited with different kinetics onto the cccDNA in cells replicating HBV imply that cccDNA bound histones may undergo regulatory post-translational modifications.

4. cccDNA quantification and activity in HBV chronic carriers

The development of highly selective real-time polymerase chain reaction (PCR) assays has provided new sensitive tools to investigate the replicative activity [50,51] and the effectiveness of antiviral therapy [28,30,45] in infected patients by enabling accurate determination of serological and intrahepatic HBV-DNA levels, including specific detection and quantification of the cccDNA in human liver biopsies. Studies have shown that cccDNA loads vary significantly in the different phases of CH-B infection and that patients who underwent HBeAg seroconversion were more likely to have lower cccDNA levels at the beginning of therapy [45]. The lower viremia generally observed in HBeAg-negative patients appeared to be due not only to the lower cccDNA content (>1 log) but also to reduced replication efficiency, which was defined as the amount of relaxed circular HBV DNA (rcDNA) produced per cccDNA molecule [51]. Quantitative measurements of HBV RNA transcripts also indicated that lower steady-state levels of pgRNA found in HBeAg-negative patients accounted for the reduced virion productivity observed at the DNA level.

The good correlation observed between pgRNA and rcDNA copy number indicated that impairment of replicative activity in HBeAg-negative patients was not due to differences in reverse transcription efficiency [50,51]. Surprisingly, impairment in virion productivity was not linked to the presence of common PC/BCP variants, since HBV replicative activity was mostly reduced in HBeAg-negative patients without detectable PC/BCP mutations [51]. Although viral titres correlated with the amount of cccDNA present in the liver in HBeAg-positive patients, there was poor correlation between viremia and intrahepatic cccDNA amounts detected in HBeAg-negative patients, implying that viral productivity can be specifically impaired in the low replicative HBeAg-negative phase of chronic hepatitis B, and strengthen the notion that viremia measurements in HBeAg-negative patients are not good predictive markers of infection in the liver [28,51]. The finding that levels of preS/S RNA transcripts and HBsAg concentrations per cccDNA molecule did not differ significantly between the two HBeAg groups, implied that only the replicative pathway was specifically impaired in those HBeAg-negative patients and hence, that the production of viral and subviral particles may be differently regulated in the course of HBV-chronic infection [51].

Nevertheless, the molecular mechanisms responsible for the lower HBV replicative activity determined in treatment naïve HBeAg-negative patients remain to be elucidated.

5. Factors regulating cccDNA activity

Due to the narrow host range of HBV and limited animal models available [52], knowledge of the factors regulating the transcriptional activity of the cccDNA *in vivo* is still very limited. These factors need to be elucidated since they will affect the size and ultimately the stability of the cccDNA pool, and therefore, will influence the clinical course of ongoing infection.

5.1. Immune-mediated factors

Experiments with HBV-replicating transgenic mice and chimpanzees have shown that inflammatory cytokines, such as type 1 interferon and TNF alpha, can effi-

ciently suppress viral replication through non-cytolytic, immune-mediated mechanisms, which also contribute to diminish the cccDNA reservoirs from infected cells [53]. However, in the chronic phase these processes may be ineffective, and in the absence of cell division, the long half-life of the hepatocytes will guarantee cccDNA survival in infected hepatocytes. Kupffer cells and dendritic cells play a major role in mediating both innate and adaptive immune responses and the molecular mechanisms by which the innate and adaptive immune response can affect viral replication are only starting to emerge [53–56]. A growing body of evidence indicates that several viruses evolved mechanisms to directly suppress the host defence by altering different pathways of the innate immune response [57,58]. Notably, chronic hepatitis B infection has been associated with a significant reduction of dendritic cell functions and impairment of the innate immune response [59]. Down-regulation of Toll-like receptor signaling was recently demonstrated in the liver and blood of HBeAg-positive chronic carriers, revealing the occurrence of potentially important interactions between virions, HBeAg and the innate immune response [56,60, Dandri, unpublished observations].

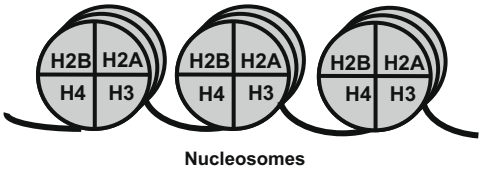
5.2. Virological factors

The complex interplay between virus and host factors occurring in the various phases of chronic infection is likely to promote the selection of certain HBV variants. A recent *in vivo* virological study [61] demonstrated ongoing significant accumulation of viral genetic diversity in HBeAg seroconverters due to selective pressure operated by the immune response. Transcription of pregenomic RNA is under the control of the basal core promoter (BCP) and mutations in this region and in the precore (PC) region are common in HBeAg-negative individuals and have been shown to affect viral replication *in vitro* [62–64]. Occurrence of mutations within the regulatory regions of the virus may also affect HBV replication activity *in vivo*. However, current knowledge of the transcriptional state of different HBV isolates is mostly based on measurements performed *in vitro* after transfection of the genomic HBV-DNA inserted on a plasmid backbone. In these systems, structure and organization of the cccDNA cannot be recapitulated, and hence, the factors influencing the transcriptional activity of the cccDNA need to be assessed *in vivo*.

Recent studies have shown that in a minority of HBeAg-negative patients only cccDNA molecules harbouring PC/BCP mutations were detected. These individuals displayed significantly higher levels of replicative activity (median 150 vs. 6 rcDNA/cccDNA) and higher mutation frequency (0.46% vs. 0.18%; $p = 0.001$) compared to HBeAg-negative patients without detectable PC/BCP mutations [65]. Of note, the selection of BCP mutations was associated with higher replication levels and worsening of clinical manifestations [51].

5.3. Epigenetic factors

Transcriptional regulation of gene expression rely on nuclear enzymatic activities that constantly modify chromatin to make it competent or refractory to gene activation. The amino-terminal tails of the four core histones on the nucleosome surface are subjected to a variety of enzyme-catalyzed, posttranslational modifications of selected amino acids, including lysine acetylation, lysine and arginine methylation, serine phosphorylation, and attachment of the small peptides ubiquitin and SUMO, that form a "histone code" specifying patterns of gene expression. Histone acetylation has long been associated with transcription initiation, whereas reduced acetylation of histones H3 and H4 N-terminal tails is a key signal for the subsequent recruitment of histone methylases and DNA methylation enzymes that results in chromatin based repression of gene expression and hetero-chromatinization [66–68]. The principal histone tails and DNA modifications and their functional impact on transcription are outlined in Fig. 3. In all eukaryotes protein coding genes transcribed by the Pol II are under the control of short discrete DNA elements arranged in promoters and enhancers. These sequences, and their complex embricate juxtapositions, dictate the binding of sequence-specific transcription factors (TFs) to activate, or repress, transcription in a gene-specific way. A common set of "general transcription farctors" (GTFs) are involved in the activation of essentially all genes. Among these, of primary importance is TBP and the associated TAFs, forming the TFIID complex. The accessibility of TFs to their target DNA sequences is regulated by the activity of a number of non-DNA binding coactivators, such as p300, CBP, PCAF/GCN5, CARM1, that (a) almost invariably harbor enzymatic activities to modify the histone tails in the nucleosomes, (b) form large >1 MDa-complexes with 10 or more subunits (SAGA, TFTC, Ccr4-Not) and (c) mediate the interplay between the gene specific factors and the general transcriptional machinery [66–68]. Since HBV cccDNA has a nucleosomal organization in infected cells and it is the template for cellular polymerases to transcribe all viral mRNAs and the RNA pregenome acetylation and deacetylation of cccDNA-bound histones might regulate transcription of viral chromatin and, by inference, viral replication. Using an antiacetylated-H3 or -H4 cccDNA ChIP assay we found that HBV replication is indeed regulated, both in cell based replication systems [44,47] and in the liver of HBV chronically infected patients [44] by the acetylation status of H3/H4 histones bound to the viral



Chromatin modifications		
Mark (either DNA or histones)	*Site*	*Function*
DNA methylation		
Methylated cytosine (meC)	CpG islands	Repression
Histone Post-Translational modifications		
Acetylated lysine (K Ac)	H3 (9, 14, 18, 56) H4 (5, 8, 13, 16) H2A, H2B	Activation
Phosphorylated serine/threonine (S/T)	H3 (3, 10, 28) H2A, H2B	Activation
Methylated arginine (R Me)	H3 (17, 23), H4 (3)	Activation
Methylated lysine (K Me)	H3 (4, 36, 79) H3 (9, 27), H4 (20)	Activation Repression
Ubiquitylated lysine (K Ub)	H2B (120) H2A (119)	Activation Repression
Sumoylated lysine (K Su)	H2B (6/7), H2A (126)	Repression

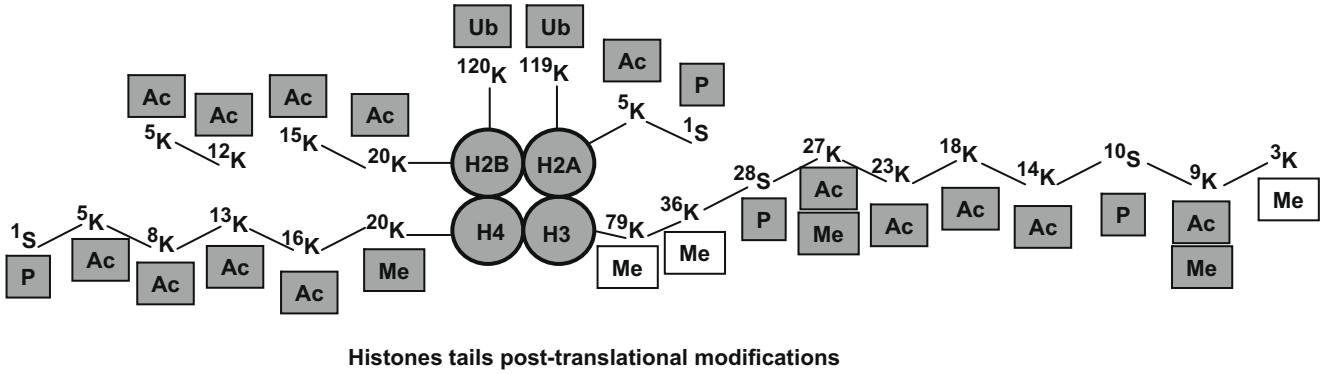


Fig. 3. Chromatin modifications and transcription. (Upper left panel) Schematic representation of nucleosomes. (Upper right panel) Type, site and function of known chromatin marks. Numbers within brackets identify the aminoacid residues involved in specific modifications. (Lower panel) Schematic representation of a nucleosome with the more frequent histone modifications.

cccDNA in the nuclei of HBV-infected cells. The co-recruitment histone acetyltransferases (PCAF and p300/CBP) parallels viral replication *in vitro* [44,48] whereas histone deacetylase 1 (HDAC1) recruitment onto the cccDNA correlate with low HBV replication *in vitro* and with low viremia *in vivo* [44] (Fig. 4). The importance of cccDNA histone epigenetic modifications in the regulation of the viral transcription/replication cycle is further confirmed by the observation that abrogation of PCAF expression by specific siRNAs reduces HBV replication and acetylation of cccDNA bound histones whereas the class I and class III histone deacetylases inhibitors trichostatin (TSA), valproate (VPA) and nicotinamide (NAM) induce an evident increase of both cccDNA-bound acetylated H4 and HBV replication [44,49]. Interestingly, valproate, that is used clinically as an anticonvulsant and more recently in the treatment of bipolar syndromes, has been shown to also increase BPV, CMV and HIV viral replication *in vitro*, to reactivate lytic replication of latent HHV8 in primary effusion lymphoma (PEL) cells [69] and FDA has recently raised a warning for the use of the drug in CMV or HIV infected patients. Although VPA has been frequently associated with hepatoxicity and severe acute hepatis this has been mainly attributed to a direct mitochondrial toxicity and the possible interaction with a co-existing HBV infection has not been assessed. By coupling cccDNA quantification and the cccDNA ChIP assay we also found that HBV genotype (A vs. D) has no effect on the accumulation of cccDNA whereas the YMDD mutant displays a reduced cccDNA accumulation [44]. Interestingly, the acetylation of cccDNA-bound H4 histone is not affected either by the HBV genotype or by the presence of the YMDD mutation, suggesting that the YMDD mutant has a defect in cccDNA molecules accumulation but the cccDNA itself is then properly assembled as a minichromosome [44]. In addition, we confirmed that anti-polymerase drugs, notably lamivudine and adefovir, do not have a noticeable effect on the accumulation of cccDNA (cccDNA molecules per cell) and on the acetylation of cccDNA-bound histone acetylation [44]. On the contrary, in response to IFNα HDAC1 is actively recruited onto the HBV minichromosone and cccDNA-bound histone

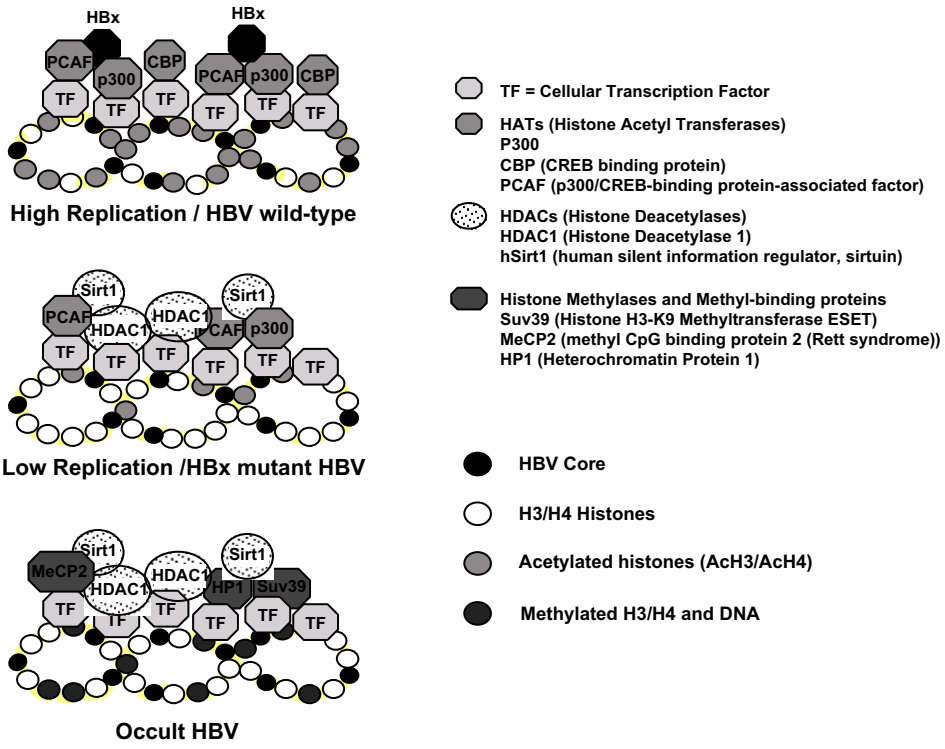


Fig. 4. Schematic representation of cccDNA-bound histones acetylation status and the recruitment of chromatin modifying enzymes onto the viral minichromosome in relation to viral replication and infection phase [40,44,49,70].

acetylation sharply decreases, without observing, in these short-term cell culture-based experiments, significant changes in the levels of cccDNA [70]. These observations suggest that the ability of class I interferon to inhibit HBV replication may be mediated, in addition to the known post-translational mechanisms and degradation of HBV transcripts [71,72], also by the epigenetic modulation of the cccDNA function. We have also shown that the HBx regulatory protein produced in HBV replicating cells is recruited onto the cccDNA minichromosome. HBV mutants that do not express HBx are impaired in their replication and that exogenously expressed HBx trans-complements the replication defects [49,73]. The kinetics of HBx recruitment on the cccDNA parallels HBV replication and is similar to that of the PCAF/GCN5 acetyltransferase [49]. Despite this observation and the physical interaction between the two proteins, we could not find any significant change in the recruitment of PCAF on the cccDNA in cells replicating the HBx defective virus. Instead, we found that cccDNA-bound histones are more rapidly hypo-acetylated in cells replicating the HBx mutant and the recruitment of the p300 acetyltransferase is severely impaired whereas the recruitment of the histone deacetylases hSirtl and HDAC1 is increased (Fig. 4) and occurs earlier. Accordingly, we show that in cells replicating the HBx mutant the pool of cccDNA is not reduced but the HBx mutant cccDNA transcribes significantly less pgRNA [49].

In addition to post-translational modifications of histones DNA methylation of CpG islands also contribute to regulate gene expression. In silico analysis of HBV genotype A identifies two CpG islands in proximity to the HBV surface gene start codon (island 1) and to the enhancer 1/X gene promoter region (island 2) [74]. The HBV-DNA extracted from circulating virions does not display significant methylation whereas the HBV CpG islands are methylated in transfected HepG2 cells and in about 50% of HBV-infected liver tissues [74]. Although this study does not specifically analyze the HBV cccDNA methylation status it shows that HBV-DNA can be methylated in human tissues and thus DNA methylation may also play a role in the regulation of HBV gene expression.

6. cccDNA is "suppressed" in occult hepatitis B

Stability and long-lasting persistence of HBV cccDNA in the nuclei of hepatocytes represent the molecular basis of occult hepatitis B infection (OBI), a form of HBV infection characterized by the persistence of HBV-DNA in the liver (and in some cases also in the serum) of individuals who test negative for the HBV surface antigen (HBsAg) [74]. On the basis of the HBV antibody profile, OBI has been distinguished in seropositive-occult hepatitis B (when anti-HBc and/or anti-HBs are present) and seronegative OBI (when anti-

HBc and anti-HBs are absent) [75]. In a small number of these individuals, failure to detect HBsAg and occult infection are due to HBV variants either producing an antigenically modified HBsAg that is not recognized by commercially available detection assays, or carrying mutations in the HBV regulatory regions or in the Pol gene responsible for defective protein synthesis and/or replication activity [76–79]. Extensive molecular analyses of entire viral genomes have revealed that occult HBV populations have a large intra-individual genetic heterogeneity that does not differ from what is found in HBsAg-positive subjects [80–82]. In most cases viral genomic variability does not appear to play a fundamental role in inducing the occult HBV status and the majority of occult HBV cases are infected with replication-competent HBVs displaying a strong suppression of replication activity and gene expression with extremely low levels of virion production [80,81]. In fact, *in vitro* functional analysis showed that occult viral isolates "re-acquire" normal capacities of replication, transcription and protein synthesis once extrapolated from the host's liver microenvironment [81]. In addition, several molecular virology studies have demonstrated the presence of both HBV cccDNA molecules and viral transcripts (including the viral pregenome) in the liver of occult infected individuals who may have or may not have HBV-DNA in the serum [81,83–85]. Real-time PCR analysis has shown median intrahepatic HBV cccDNA levels about 1 log lower than those observed in HBeAg-negative chronic HBV carriers (0.002 copies/cell vs. 0.01 copies/cell) [45]. The persistence of such minute amounts of cccDNA is consistent with the hypothesis that the clinical resolution of HBV infection does not occur through a complete eradication of the virus from the liver and rather viral replication is controlled by the host's immune system. The presence of a complete HBV replication cycle in HBsAg-negative cases is confirmed both by the demonstration of HBV-specific T-cell responses in patients with occult HBV infection, even when serum HBV markers were completely negative [86] and by the observations that occult HBV can be transmitted through blood transfusion or organ transplantation and occult HBV carriers are at risk of HBV reactivation when exposed to immune suppression (i.e. hematologic malignancies, HIV infection, cancer chemotherapy, etc.) [87,88]. The molecular mechanisms that keep viral replication under control in seronegative occult infections have not been fully defined. Innate immune response and/or interference by coinfecting agents (i.e. hepatitis C virus) may contribute as additional factors negatively influencing HBV replication and gene expression. However, according to our findings on the important role played by chromatin remodelling enzymes and the epigenetic modulation of cccDNA function we have found that the profound suppression of viral replication and viral transcription in OBI livers gene associates with the recruitment of several cellular several histone deacetylases and histone methylases onto the HBV minichromosome (Pollicino, personal observations) (Fig. 4) and with a specific pattern of HBV-DNA methylation in occult infected patients [82].

7. Conclusions

The availability of sensitive molecular assays for direct cccDNA quantitation in liver tissues and the development of new techniques based upon the analysis of chromatin dynamics to study the regulation of cccDNA function both in cellular models of HBV replication and *in vivo* has significantly advanced our knowledge of the molecular mechanisms regulating HBV replication and persistence. One major conceptual advance has been the recognition of the existence of a complex network of epigenetic events that influence both cccDNA function and HBV replication. Although more work is needed to expand our knowledge, results suggest that inactive carriers, anti-HBe positive patients during low replication phases and patients with occult HBV infection may share, although to a different degree, common mechanisms of active cccDNA suppression mediated by epigenetic modifications of cccDNA and cccDNA-bound histones. The feasibility to modulate in a cell culture model cccDNA transcription using zinc finger proteins (ZFPs) engineered to specifically bind and repress the activity of the DHBV enhancer has been recently reported [89]. Although attractive, in principle this approach might be limited by the need for ZFPs to be delivered using viral vectors or included in liposomes or nanoparticles designed in order to target the infected liver. The identification of epigenetic marks of active and inactive cccDNA and the precise definition of the cellular enzymatic activities modulating transcription of the cccDNA minichromosome, as well as the cellular signaling pathways that modulate their activity, opens new perspectives for the identification of relevant therapeutic targets to permanently suppress HBV transcription/replication. On the other hand, we must continue our efforts to understand the molecular basis of cccDNA stability. Relevant issues would include: (a) the identification of the cellular proteins that are involved in the conversion of RC DNA into the cccDNA; (b) the identification of proteins and pathways affecting the size of the nuclear HBV cccDNA pool in different categories of HBV patients; (c) the comparison of naked vs. chromatinized cccDNA half-life to elucidate whether open/transcribed cccDNA would be less stable compared to packed/inactive cccDNA. This knowledge will hopefully allow us, in the long term, to move a step forward from the control of HBV replication and disease to the cure of HBV infection.

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