ORIGINIAL RESEARCH

HIRA Supports Hepatitis B Virus Minichromosome Establishment and Transcriptional Activity in Infected Hepatocytes

Maëlle Locatelli,1,2 Jean-Pierre Quivy,3,4 Fleur Chapus,1,2 Maud Michelet,1 Judith Fresquet,1 Sarah Maadadi,1 Amel Neila Aberkane,1,2 Audrey Diederichs,1 Julie Lucifora,1,2 Michel Rivoire,5 Genevieve Almouzni,3,4 Barbara Testoni,1,2,§ and Fabien Zoulim1,2,6,§

1INSERM U1052, CNRS UMR-5286, Cancer Research Center of Lyon, Lyon, France; 2University of Lyon, Université Claude-Bernard, Lyon, France; 3Institut Curie, PSL Research University, CNRS, UMR3664, Equipe Labellisée Ligue Contre le Cancer, Paris, France; 4Sorbonne Universités, UPMC Université Paris 06, CNRS, UMR3664, Paris, France; 5Oncology Surgery Department, Centre Léon Bérard, Lyon, France; 6Hospices Civils de Lyon, Lyon, France

SUMMARY
We describe the involvement of the histone chaperone histone regulator A in the formation of the chromatinized form of hepatitis B virus episome in the nucleus of infected hepatocytes. Histone regulator A also is required for full transcriptional activity of the established hepatitis B virus minichromosome.

BACKGROUND & AIMS: Upon hepatitis B virus (HBV) infection, partially double-stranded viral DNA converts into a covalently closed circular chromatinized episomal structure (cccDNA). This form represents the long-lived genomic reservoir responsible for viral persistence in the infected liver. Although the involvement of host cell DNA damage response in cccDNA formation has been established, this work investigated the yet-to-be-identified histone dynamics on cccDNA during early phases of infection in human hepatocytes.

METHODS: Detailed studies of host chromatin-associated factors were performed in cell culture models of natural infection (ie, HepG2NTCP cells) and primary human hepatocytes infected with HBV, by cccDNA-specific chromatin immunoprecipitation and loss-of-function experiments during early kinetics of viral minichromosome establishment and onset of viral transcription.

RESULTS: Our results show that cccDNA formation requires the deposition of the histone variant H3.3 via the histone regulator A (HIRA)-dependent pathway. This occurs simultaneously with repair of the cccDNA precursor and independently from de novo viral protein expression. Moreover, H3.3 in its S31 phosphorylated form appears to be the preferential H3 variant found on transcriptionally active cccDNA in infected cultured cells and human livers. HIRA depletion after cccDNA pool establishment showed that HIRA recruitment is required for viral transcription and RNA production.

CONCLUSIONS: Altogether, we show a crucial role for HIRA in the interplay between HBV genome and host cellular machinery to ensure the formation and active transcription of the viral minichromosome in infected hepatocytes. (Cell Mol Gastroenterol Hepatol 2022; ■■■■; https://doi.org/10.1016/j.jcmgh.2022.05.007)
Keywords: HBV; cccDNA; chromatin; HIRA; H3.3.

Hepatitis B virus (HBV) is a hepadnavirus that necessitates a mandatory nuclear phase to convert the partially double-stranded relaxed circular DNA (rcDNA) covalently attached to the viral polymerase protein contained in the virion into a covalently closed circular DNA (cccDNA) episome.\(^1\) cccDNA represents not only the unique transcriptional template for viral protein expression and genome replication, but also a long-lived repository for viral genetic information. Similar to other DNA viruses that persist as episomes in host nuclei, the HBV genome must adopt a chromatin structure to maintain the stability and accessibility of its genome and thus relies on host cellular molecular machinery for its replication.\(^2,3\) HBV can be assimilated to a pararetrovirus because viral genome replication requires the reverse transcription of the pre-genomic RNA (pgRNA) into new rcDNA by the viral polymerase within newly assembled nucleocapsids in the cytoplasm.\(^1\) Unaffected by current antiviral treatments inhibiting viral DNA synthesis, cccDNA is responsible for viral persistence in infected hepatocytes and its elimination remains the utmost goal for curing chronic HBV infections.\(^4\)

Unlike SV40 and papilloma viruses, which already package their circular DNA genomes in a nucleosomal state in the virion,\(^5\) HBV rcDNA enters the host cell nucleus devoid of nucleosomes and must divert the host cell machinery to build a de novo chromatin structure in the newly infected cells. A number of recent studies have indicated that the conversion of rcDNA into cccDNA necessitates events linked to DNA repair.\(^6-13\) However, the factors involved in histone deposition on the nucleosome-free HBV genome in the hepatocyte nuclei have been largely overlooked to date.

The histone regulator A (HIRA) promotes H3.3 deposition and nucleosome assembly independently of DNA synthesis by exploiting its capacity to bind nonspecifically to H3.3-H4 histone dimers and naked DNA.\(^14-16\) HIRA recently was involved in the deposition of H3.3 onto foreign herpes simplex virus and cytomegalovirus (CMV) genomes.\(^17\) H3.3 histone variant is expressed constitutively throughout the cell cycle in proliferating and quiescent cells. It is distinguished from the replicative variants H3.1/2 by a single amino acid substitution in the amino-terminal tail, a serine at position 31 in place of an alanine in H3.1/2 and in the regions recognized by distinct chaperones.\(^18\) The importance of phosphorylation at S31 in H3.3 has been shown recently for major transitions in transcription programs.\(^16-20\)

In this study, we analyzed the cccDNA nucleoprotein structure in human hepatocytes very early after de novo infection. Both histone deposition on incoming HBV genome and viral transcript expression began within hours after viral entry and the histone chaperone complex HIRA-dependent H3.3 deposition was essential for the formation of supercoiled cccDNA. Although HBV protein neosynthesis was dispensable for HIRA and H3.3 recruitment to cccDNA, the viral core protein delivered by incoming virions was temporarily and physically associated to cccDNA-bound HIRA. The observation that HIRA depletion after the establishment of cccDNA severely decreased the expression of viral RNA uncovered a dual role for HIRA complex in both HBV chromatin assembly and transcriptional activation by promoting deposition and recycling of serine 31 phosphorylated H3.3.

Results
Supercoiled and Transcriptionally Active cccDNA Appears Early After Viral Infection

Kinetics of cccDNA formation in Hepg2\(^{\text{NTCP}}\) cells and in primary human hepatocytes (PHHs) were analyzed by Southern blot and quantitative polymerase chain reaction (qPCR) after infection with HBV inoculum in the presence or not of a specific entry inhibitor that served to set the detection background. To specifically enrich for viral episomal genome in the nucleus of infected hepatocytes, an adapted Hirt extraction procedure was performed to select for genomic forms already deprived of the covalently attached viral polymerase.\(^21\) This viral genome population presumably consists of a mixture of at least 3 distinct DNA species, one with both strands remaining open; another with a covalently closed minus strand rcDNA, collectively protein-free rcDNA (PF-rcDNA); and the double-strand covalently closed cccDNA.\(^22,23\) cccDNA can be specifically distinguished from the other species by virtue of the difference in electrophoresis mobility associated to its supercoiled conformation (Figure 1A and B). The incoming viral genome was comprised mostly of PF-rcDNA (Figure 1B) (12 hours postinfection [hpi] and entry inhibitor treated samples), while cccDNA appeared afterward (16 hpi). Interestingly, PF-rcDNA levels decreased along with a cccDNA increase from 48 to 72 hpi, in agreement with its role as a direct precursor of cccDNA formation\(^24\) (Figure 1A). Consistently, using the more sensitive real-time qPCR method, cccDNA was detected as soon as 2 hpi and began to increase at 4 hpi (Figure 1C and F). The levels of intracellular 3.5-kb HBV RNA, detecting the replicative intermediate pgRNA, increased exponentially from 24 hpi, indicating the initiation of viral transcription from cccDNA.

© 2022 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0).
Figure 1. HBV minichromosome establishment occurs very rapidly after infection. (A and B) Southern blot analysis of cccDNA appearance kinetic. PHHs or HepG2\(^{\text{NTCP}}\) cells were infected with HBV in the presence or not of 100 nmol/L preS1-mimicking peptide for up to 16 hours and then harvested at the indicated times points. Mitochondrial DNA was used as an internal loading control. The specificity of the cccDNA band is shown by linearization after digestion with EcoRI or XhoI restriction enzymes. (C-H) qPCR quantification of viral cccDNA, 3.5-kb RNA, and total HBV DNA (tHBV-DNA) in (C–E) PHHs and (F–H) HepG2\(^{\text{NTCP}}\)-infected cells. Cells were inoculated with HBV for up to 16 hours and then harvested at the indicated time points. cccDNA and tHBV-DNA quantification were normalized over \(\beta\)-globin quantity, while the relative 3.5-kb RNA amount was normalized over the housekeeping gene GUS\(\beta\) expression. Graphs represent the means ± SEM of at least 3 independent experiments. MW, ______; p.i., ______.
Quantification of total HBV DNA, including rcDNA delivered by incoming virions, cccDNA and replicative intermediates generated by pgRNA reverse transcription, showed a basal level as a result of the input virions entering the cells that remained stable until 48 hpi, followed by an increase indicating the initiation of pgRNA reverse transcription and active viral replication (Figure 1E and H). Taken together, these data show that PF-rcDNA to
ccDNA conversion occurs early after infection and precedes a phase in which it occurs concomitantly with viral transcription (from 24 hpi onward) of established ccDNA pool and total DNA accumulation (from 48 hpi onward) with similar kinetics in HepG2\textsuperscript{hNTCP} cells and primary human hepatocytes.

**HIRA Is Required for Full ccDNA Supercycling in De Novo Infected Cells**

De novo histone deposition is required to form nucleosomes on the naked ccDNA precursor.\(^{25}\) A number of biological reactions are required to convert partially double-stranded, viral polymerase-associated rcDNA into ccDNA and most of them could be ascribed to DNA repair pathways.\(^{26}\) Interestingly, histone H3 variants H3.1/2 and H3.3 and their respective histone chaperone complexes, chromatin assembly complex (CAF-1) and HIRA, have been involved in histone deposition linked to DNA damage,\(^{27,28}\) placing them as attractive candidates to promote nucleosome assembly on ccDNA. However, after dimethylosulfide (DMSO) treatment, which favors cell differentiation and viral infection, while HIRA levels remained stable, expression levels of the p150 subunit of CAF-1 decreased in nondividing HepG2\textsuperscript{hNTCP} (Figure 2A), as already observed for other differentiated cells.\(^{29}\) These data, along with the observation that only HIRA could deposit H3.3 on foreign viral DNA,\(^{17}\) prompted us to investigate the contribution of HIRA in ccDNA formation within the first 72 hpi in HepG2\textsuperscript{hNTCP} cells. Two sequential small interfering RNA (siRNA) transfections (Figure 2B) were performed to achieve a significant reduction of HIRA expression, both at the messenger RNA and protein levels (Figure 2C and D), without significant effects on cell viability (Figure 2E) and HBV-receptor hNTCP RNA and protein expression (Figure 2F and G). HBV infection was performed 48 hours after the second transfection, when HIRA protein was depleted more than 70% (Figure 2C and D). Southern blot and qPCR analysis at 48 hpi showed a sharp decrease of ccDNA amounts in HIRA-depleted cells compared with controls (Figure 2H and I). Importantly, HIRA knockdown did not affect the levels of PF-rcDNA, suggesting that HIRA is not required for processes preceding PF-rcDNA to ccDNA conversion, but is necessary for the subsequent steps leading to ccDNA formation and maintenance (Figure 2H).

**A Naked, Not Fully Double-Stranded Precursor Is the Substrate of HIRA During ccDNA Formation**

Next, we asked whether requirement for HIRA is linked to repair events associated with conversion of PF-rcDNA toward ccDNA (ie, a complete circular double-stranded DNA). First, we verified if the levels of transcripts of genes involved in rcDNA to ccDNA conversion\(^{20,21}\) were affected upon loss of HIRA. We could not show significant differences in POLK, EXO1, tyrosyl-DNA phosphodiesterase-2, and flap structure-specific endonuclease 1 transcript expression (Figure 3A–D) after HIRA silencing and trans-complementation, suggesting that repair activities involved in the first processing events of HBV genome are not affected by HIRA down-regulation. Next, we monitored kinetics of bromodeoxyuridine (Brdu) incorporation in the nuclear HBV genome to assess the DNA repair activity during the early phases of HBV infection by performing ccDNA–chromatin immunoprecipitation (ChIP) analysis using an anti-Brdu-specific antibody (Figure 3E). Brdu incorporation was detected in ccDNA at 48 and 72 hpi indicating that repair and “gap filling” processes are involved in ccDNA formation in living cells (Figure 3E). Taking advantage of the minicircle technology,\(^{29}\) we introduced nicks in 1 strand of the circular double-stranded minicircle HBV (mchBV) by digestion with the nicking endonuclease Nb.BsrDI, triggering mchBV relaxation and mimicking a covalently closed minus strand rcDNA structure, recently described as the main precursor of ccDNA\(^{22,23}\) (Figure 3F). After HIRA knock-down, either a double-stranded or a nicked mchBV was transfected and the formation of supercoiled ccDNA was monitored by Southern blot (Figure 3F). The levels of ccDNA arising from the double-stranded circular mchBV were not affected by the absence of HIRA. In contrast, when the damaged mchBV was transfected, less supercoiled ccDNA formation was detected in the absence HIRA (Figure 3F). This suggests that HIRA is critical for ccDNA formation when DNA repair is required, such as conversion of PF-rcDNA after its entry in the nucleus of infected cells.

**HIRA-Dependent ccDNA Formation Does Not Require HBV Protein Neosynthesis**

To investigate a putative role of early translated HBV proteins in HIRA-dependent ccDNA formation, we took advantage of an HBx-deficient HBV virus (ΔHBx-HBV).\(^{30}\)

---

**Figure 2.** (See previous page). Full ccDNA supercycling in de novo infected cells requires HIRA protein expression. (A) Detection by Western blot analysis of CAF-1 (subunits p150 and p60) and HIRA before and after 72 hours of 2.5% DMSO addition to HepG2\textsuperscript{hNTCP} cells. (B) HepG2\textsuperscript{hNTCP} cells were transfected twice with siRNA anti-HIRA or a nontargeting siRNA (siCTRL) and then inoculated for 16 hours with HBV. Cells were harvested for analysis 2 dpi. (C and D) HIRA messenger RNA (mRNA) and protein expression after siRNA transfection was determined by (B) real-time qPCR and (C) Western blot, respectively. β-actin served as Western blot loading control. HIRA mRNA was normalized over housekeeping GUSB gene levels and expressed as relative to the control condition treated only with the transfection reagent (TRA). (E–G) HepG2\textsuperscript{hNTCP} cells were transfected with siRNA against HIRA according to the timelines shown in Figure 6A and inoculated for 16 hours with HBV at 250 viral genome equivalents/cell. The cells were harvested for analysis 2 dpi. (E) Neutral red and sulfanilamide cytotoxicity assay analysis. Doxorubicin treatment served as positive control for cell mortality. (F) mRNA and (G) protein levels of hNTCP receptor. β-actin served as Western blot loading control. (H and I) ccDNA amount at 2 dpi was measured by (H) Southern blot and (I) qPCR. ccDNA quantification was normalized over β-globin quantity and expressed as relative to TRA control. p.i., ____.
Figure 3. Full cccDNA supercoiling in de novo infected cells occurs concomitantly to HBV genome repair. (A–D) Messenger RNA levels of (A) POLK, (B) EXO1, (C) TDP2, and (D) FEN1 were quantified by real-time qPCR assay and expressed as a percentage of TRA-treated cells after normalization over GUSB housekeeping gene expression. Data represent the means ± SEM of at least 3 independent experiments. (E) ChIP analysis of BrdU-containing cccDNA molecules. HepG2 cells were treated with 20 μmol/L for 24 hours before HBV infection and cultured for the indicated time points. cccDNA-ChIP qPCR using no antibody or anti-E2F antibody served as ChIP technical negative controls (Figure 8A and B), and the signal at 0.5 hpi was considered aspecific qPCR background for cccDNA quantification (Figure 1F). Data are expressed as a percentage of enrichment with respect to initial input chromatin. (F) mcHBV constructs digested or not with Nb.BsrDI were transfected into HepG2 cells 48 hours after transfection with siHRA or siCTL. Cells were harvested 24 hours after mcHBV transfection and the cccDNA amount was measured by Southern blot. Graphs represent the means ± SEM of at least 3 independent experiments. The 2-tailed P value was calculated for a risk threshold of .05 using the 2/K sample permutation test with Monte Carlo resampling approximation. *P < .05, **P < .01, and ***P < .001. EXO1, ______; FEN-1, flap structure-specific endonuclease 1; OC (rc)-HBV DNA, open circular (relaxed circular) HBV DNA; POLK, ______; TDP2, tyrosyl-DNA phosphodiesterase-2.
The HBx protein plays a pivotal role for the full transcriptional activity of established cccDNA, thus HBx-HBV infection allows the formation of cccDNA, which, however, remains transcriptionally inactive.30,31 After infection with a HBx-HBV genome containing virus, cccDNA appeared with similar kinetics as WT-HBV virus, while no 3.5-kb RNA and DNA replicative intermediates could be detected in PHHs (compare Figure 1C–E with Figure 4A–C) and HepG2NTCP (compare Figure 1F–H with Figure 4D–F). HIRA knock-down before HBx-HBV infection decreased cccDNA levels to a similar extent as in WT HBV infection conditions in PHHs (Figure 4G) and HepG2NTCP (Figure 4H).

In experimental models of nuclear import,32,33 the naked nucleocapsids were shown to enter the nuclear pore to release the rcDNA, together with the viral capsid protein (HBc), into the nucleoplasm. Nonetheless, the role of HBc in cccDNA formation, if any, remains to be elucidated. First, we investigated the kinetics of HBc binding to cccDNA by ChIP-qPCR experiments, finding that HBc was bound to cccDNA at all time points investigated (Figure 5A). The binding was not affected when infection was performed with a HBx mutant genome deficient for de novo viral protein expression, thus implying that HBc from the viral inoculum migrated to the nucleus and bound to cccDNA at early time points after infection.
infection (Figure 5B). Sequential ChIP experiments performed 24 hpi in HepG2<sub>N</sub>TCP cells indicated that HBC and HIRA could bind to the same cccDNA molecule (Figure 5C).

This co-occupancy is in line with proximity ligation assay (PLA) experiments evidencing a high proximity between the 2 proteins in HBV-infected PHHs at 24 hpi (Figure 5D).
Figure 6. HIRA trimerization is required for HBV minichromosome establishment. (A) HepG2hNTCP cells were transfected twice with siHIRA or siCTL and then transfected with plasmids encoding for either WT HIRA (pEYFP-N1-HIRA) or for a trimerization-incompetent HIRA mutant (pEYFP-N1-HIRA W799A D800A) before inoculation with HBV. The cells were harvested for analysis 2 dpi. (B) HIRA messenger RNA (mRNA) and protein expression after siRNA transfection and transcomplementation was determined by real-time qPCR and Western blot. β-actin served as Western blot loading control. HIRA mRNA was normalized over housekeeping GUSb gene expression and expressed as relative to the control treated only with the TRA. (C) cccDNA levels at 2 dpi were measured by qPCR. The cccDNA amount was normalized over β-globin quantity and then expressed as relative to the control treated only with TRA. (D) Schematic representation indicating that HIRA trimerization, required for H3.3 deposition, is necessary for full PF-rcDNA to cccDNA conversion in living infected hepatocytes. Graphs represent the means ± SEM of at least 3 independent experiments. The 2-tailed P value was calculated for a risk threshold of 0.05 using the 2/K sample permutation test with Monte Carlo resampling approximation. *P < .05, **P < .01, and ***P < .001.

Figure 5. (See previous page). Incoming HBV core protein associates to cccDNA and HIRA early after infection. (A and B) HepG2hNTCP cells were infected with either WT or ΔHBx HBV for up to 16 hours and then extensively washed and cultured for the indicated time points before ChIP analysis using an antibody against HBC. The levels of HBC on cccDNA were analyzed through the infection kinetics by ChIP–qPCR and expressed as the percentage of input chromatin. cccDNA-ChIP qPCR using no antibody (NoAb) or anti-E2F antibody served as technical negative controls (Figure 5A–D), and the signal at 0.5 hpi was considered as specific qPCR background for cccDNA quantification (Figure 1F). (C) The simultaneous presence of HIRA and HBC on the same cccDNA molecule was assessed by sequential ChIP–qPCR 24 hpi using an antibody against HIRA first and then an antibody anti-HBC for immunoprecipitation. NoAb-NoAb, NoAb-HBC, and HIRA-NoAb combinations of sequential IP served as negative controls, and IP with single HBC and HIRA served as positive controls. Graphs represent the means ± SEM of at least 3 independent experiments. (D) Proximity between HBC and HIRA was assessed by PLA in HBV-infected PHHs at 24 hpi. The PLA signal is indicated by arrows. Uninfected and infected PHHs stained with only HBC or HIRA antibodies were used as negative controls (lower panels). (E) Immunofluorescence and (F) flow cytometry analysis of HBC-positive PHHs at 24 hpi and 7 dpi. Immunofluorescence was performed with an antibody against HBC (red) and nuclei are stained by 4',6-diamidino-2-phenylindole (DAPI) signal (blue). (G) Western blot analysis of HIRA-HBC immunoprecipitation in HepaRG cells inducible for HBC expression. Immunoprecipitation was performed with an anti-HBC antibody, using HepaRG-TR-HBC noninduced cells as a control (left panel). Western blot with anti-HIRA antibody showed a specific band in the immunoprecipitated fraction in the presence of HBC (right panel).
when approximately 15% of cells are positive for anti-HBc staining (Figure 5E and F). Moreover, using a HepaRG cell line expressing HBc (HepaRG-TR-HBc), we showed that HBc and HIRA not only can be located in close proximity but also can co-immunoprecipitate (Figure 5G). Taken together, these data exclude the need of HBV protein neosynthesis for
HIRA-dependent cccDNA formation, but suggest a yet-to-be
investigated involvement of the viral capsid protein in the
eyeletetic events leading to cccDNA formation.

**HIRA Trimerization Is Required for cccDNA Formation**

To further confirm the involvement of HIRA in histone
loading during cccDNA formation, we rescued HIRA
expression after silencing in HepG2 nntcp cells by trans-
fecting a construct containing either a wild-type (WT) (pEYFP-N1-HIRA) or a HIRA mutated sequence (pEYFP-N1-
HIRA W799A D800A) 8 hours before HBV infection
(Figure 6A). The latter encodes a protein unable to homo-
trimerize, a property that is essential for new H3.3 deposi-
tion. As shown in Figure 6, reverse-transcription qPCR
and Western blot analysis confirmed the restoration of HIRA
messenger RNA levels and the appearance of the exogenous
protein isoforms upon transfection (Figure 6B). In parallel,
the cccDNA amount evaluated by qPCR went back to the
level of nonsilenced controls when HIRA depletion was
transcomplemented with wild-type HIRA, but not with the
W799A D800A HIRA mutant (Figure 6C). These data argue
for the requirement of H3.3 deposition function of the HIRA
trimer during the cccDNA formation process in the nucleus
of living hepatocytes (Figure 6D).

**Histone Variant H3.3 Is Deposited on cccDNA During Its Chromatinization**

As shown in Figure 1, the appearance of cccDNA spans at
least the first 72 hpi, precedes viral transcription by 24
hours, and overlaps with it afterward (Figure 1C, D, F, and
G). HIRA cccDNA-ChIP experiments performed between 0.5
and 72 hpi in HepG2 nntcp cells showed that HIRA associa-
tion to cccDNA was detected (ie, 2 hpi) (Figure 1F), remained
stable until 12 hpi, and then increased (Figure 7A), in par-
allel with the recruitment of RNA polymerase II (RNAPII)
on cccDNA (Figure 7B) and the beginning of viral tran-
scription (Figure 1G). Importantly, cccDNA-ChIP enrichment
appears specific, given that negative controls, including
unrelated protein E2F or lack of primary antibody, did not
generate any cccDNA signal (Figure 7I and J). HIRA
increased binding to cccDNA was not correlated to any
significant modulation of HIRA expression or localization in
infected cells compared with uninfected ones (Figure 8).
HIRA association with cccDNA thus appears to follow a 2-
step behavior with a recruitment at 2 hpi that remains
constant up to 12 hpi, followed by a sharp 6-fold increase at
24 hpi that lasts until 72 hpi. In line with HIRA ChIP signal,
H3.3 was detected associated to cccDNA, also showing a 2-
phase recruitment kinetic, with a peak at 8 hpi and then a
progressive increase beginning at 12 hpi (Figure 7C). Across
the same time points, no specific enrichment for H3.1/3.2
was detected (Figure 7C). ChIP signal from an antibody
recognizing all 3 variants (H3pan) showed a cccDNA
enrichment profile similar to that of H3.3, suggesting that
the histone variant might represent most of H3 protein
associated to cccDNA within 72 hpi. The specificity of H3.3
and H3.1/3.2 antibodies in ChIP was confirmed by analyzing
preferential recruitment of H3.3 and H3.1/3.2 to their respective
targets on host cell genome (Figure 9A–F). In parallel,
nucleosomal core histones (H2A, H2B, and H4) also
were found associated to cccDNA from 2 hpi onward
(Figure 9G–I), further indicating nucleosome assembly on
cccDNA.

**Transcription of the Established cccDNA Pool Is Associated With the Recruitment of HIRA and Phosphorylation of H3.S31**

Recent data in an artificial transfection-based model of
HBV replication indicated that exogenously expressed H3.3
affected HBV RNA levels. H3.3 tail differs from canonical
histones H3.1/H3.2 for a serine in place of an alanine in
position 31. Parameter evidence supports the possibility
that H3.3S31 could regulate through phosphorylation key
activities driving specific transcription programs. In our
model of natural HBV infection, H3.3S31p was detected
by cccDNA-ChIP from 12 hpi onward and showed an
enrichment kinetic superimposable to that of H3.3
(Figure 7C). To determine if H3.3 recruitment to cccDNA
and S31p after 12 hpi could be linked to transcriptional
activity, HepG2 nntcp cells were infected with the ΔHBx viral
strain (Figure 4). Interestingly, kinetics of recruitment of
HIRA and H3.3 did not differ significantly between WT and
ΔHBx infections within the first 12 hpi (Figure 7A, C, E, and
G) and, as expected, lack of viral transcription was associ-
ated with no specific enrichment of RNAPII on cccDNA
(Figure 7F) and the appearance of the SMCS/6 repressive
complex on cccDNA derived from ΔHBx HBV, as shown
by the recruitment of the subunit NSE4 (Figure 7H), which
was barely associated to transcriptionally active
cccDNA (WT HBV condition) (Figure 7D). However, in
contrast to WT, we did not detect increased recruitment of
HIRA and H3.3 in the ΔHBx condition, or specific H3.3S31p
after 12 hpi. Nevertheless, we could detect an increasing

**Figure 7. (See previous page). Dynamics of HIRA and H3.3 recruitment and phosphorylation of H3.3S31 during de novo cccDNA formation and transcription of established cccDNA pool.** HepG2 nntcp cells were infected with (A–D) WT or (E–H) ΔHBx-HBV for up to 16 hours and then extensively washed and cultured for the indicated time points before ChIP-qPCR
analysis using antibodies against (A and E) histone chaperone HIRA, (B and F) RNAPII, (C and G) total H3 (H3pan), and
histone variants H3.3 and H3.1/2 and H3.3S31p, and (D and H) SMCS/6 complex subunit NSE4. (I–L) cccDNA-ChIP qPCR
using no antibody (NoAb) or anti-E2F antibody served as technical negative controls. The signal at 0.5 hpi was considered
aspecific qPCR background for cccDNA quantification (Figure 1F). (M) Snap-frozen liver samples from 3 chronically infected
male patients were subjected to cccDNA-ChIP with antibodies against HIRA, H3.3, H3.3S31p, and H3.1/2. NoAb IP served as
ChIP negative control. Data are expressed as a percentage of enrichment with respect to initial input chromatin and represent
the means ± SEM of at least 3 independent experiments.
Figure 8. HIRA expression levels and cellular localization are not affected by HBV infection. HepG2\textsuperscript{hNTCP} cells or PHHs were cultured in 2.5% DMSO containing medium for 72 hours, HBV infected at 250 viral genome equivalents/cell for up to 16 hours and then extensively washed and cultured for the indicated time points. HIRA messenger RNA (mRNA) and protein expression were analyzed throughout the infection kinetic by (A) real-time qPCR, (B) Western blot, and (C and D) immunofluorescence. HIRA mRNA levels were normalized over the housekeeping gene GUS\textsubscript{b}. \beta-actin signal served as loading control for Western blot analysis. Graphs represent the means ± SEM of at least 3 independent experiments. Immunofluorescence was performed with antibodies against HIRA (green), promyelocytic leukemia protein (PML, red), and HBV S protein (violet). Nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) signal in grey.
enrichment for H3.1/3.2 recruitment to cccDNA that was not detected in the WT (Figure 7G), as if a lack of H3.3 was compensated by H3.1/2 deposition. Negative controls, including unrelated protein E2F or lack of primary antibody, did not generate any cccDNA signal, confirming the specificity of cccDNA-ChIP enrichment (Figure 7K and L). These data suggest that HIRA is involved in both cccDNA formation in the very early phases after PF-rcDNA entry into the host cell and transcription from the established viral michromosome Q30 pool via the deposition of H3.3, which was found to be phosphorylated on S31 on transcriptionally active cccDNA. This conclusion is supported by the observation that, after SMC5/6 complex knockdown and cccDNA transcription recovery, HIRA, H3.3, and H3.3S31ph recruitment to ΔHBx-HBV cccDNA was increased (Figure 10).

Notably, the association between cccDNA, HIRA, and H3.3 in its S31 phosphorylated form was confirmed in vivo in liver tissues derived from chronic HBV-infected patients (Figure 7M).

**HIRA Is Required for the Maintenance of cccDNA Transcriptional Activity**

To further confirm HIRA involvement in cccDNA transcriptional activity, we depleted HIRA once the cccDNA pool was established (ie, 4 dpi)23,37 (Figure 11A).
Discussion

The HBV cccDNA must adopt a chromatin structure similar to that of the host cell chromosomes for its transcription\(^3,38,39\) in the infected hepatocyte nuclei. Coupling Southern blot and ChIP-qPCR analysis, we found that histone loading occurred simultaneously with repair of the partially double-stranded cccDNA precursor (PF-rcDNA). Although our data were generated from a population of cccDNA containing infected hepatocytes, we can assume that the breaks into PF-rcDNA strands, evoking a target for the DNA repair machinery, represent a trigger for HIRA recruitment enabling subsequent histone deposition coupled to DNA repair, in agreement with the role of HIRA in priming damaged chromatin early after damage recognition.

Previous work conducted in cell-free systems have reproduced steps leading from PF-rcDNA to supercoiled...
cccDNA without the need for histone chaperones.\textsuperscript{8,9} Interestingly, we showed that in infected hepatocytes, a defective H3.3 deposition owing to HIRA depletion or the lack of HIRA homotrimerization severely impacted the appearance of supercoiled cccDNA. Therefore, it can be argued that nucleosome formation is not required for the conversion of PF-rCdNA to a complete double-stranded circular DNA molecule per se, but that HIRA (and H3.3 deposition) is
required for the stabilization of the HBV genome as an episome in the nucleus of living hepatocytes. This also suggests that HIRA is involved in preventing degradation of naked DNA and/or shaping the viral genome into a chromatin structure that can be handled by the host transcriptional machinery for the benefit of the viral genome. It will be interesting to determine the kinetics of events on single cccDNA molecules, when the yet unresolved technical limitations in analyzing single HBV genomes in infected nuclei will be overcome.

In our experimental conditions, from 24 hpi onward, formation of new cccDNA molecules and transcription occurred concomitantly. The use of a modified HBV lacking the regulatory HBx protein (ΔHBx) resulting in transcriptionally inactive cccDNA in infected hepatocytes provided evidence for transcription-coupled HIRA recruitment to established cccDNA and for the association between H3.3 S31 phosphorylation and active viral transcription. H3.3S31ph recently was related to increased in-cis acetylation along with a decrease of trimethylation of its lysine in position 27, thus providing chromatin access to regulatory factors at selected transcribed regions. Notably, cccDNA-associated H3 histone tails have been shown to be highly acetylated on K27, while no specific trimethylation was detected in infected hepatocytes and human liver samples.

Our results highlighted a preferential deposition of H3.3 over H3.1/2 on cccDNA in liver samples from HBV-infected patients. Whether this would be instrumental for H3.3S31ph to happen and to regulate access to the viral genome of either viral and host cell chromatin regulators warrants further investigation. The demonstration that HIRA depletion decreased H3.3, its S31 phosphorylated form, and RNA Pol II enrichment on established cccDNA and affected viral RNA production further suggests a pivotal role for HIRA in maintaining cccDNA active transcription. Interestingly, homotrimerization of HIRA does not seem to be required for its binding to established cccDNA, suggesting a possible difference in HIRA-associated protein partners during de novo H3.3 deposition vs recycling on cccDNA, as already observed on the human genome. However, we cannot rule out a possible involvement of other histone chaperons in cccDNA activity at later time points, as indicated for CAF-1 by Yang et al at 12 days after infection.

Differently from the observation made with herpes viruses and cytomegalovirus, we did not see any specific HIRA relocation to promyelocytic leukemia bodies after HBV infection either in HepG2B72 or in primary human hepatocytes (Figure 8C and D). This is consistent with the observation that HBV infection might be established without significant perturbation of host cell innate immune response. Indeed, the HBV genome remains shielded from cytoplasmic innate immune sensors thanks to its capsid shell, which shuttles the viral DNA until the inner face of the nuclear basket. Given the association of the HBV capsid protein (HBC) to cccDNA at all investigated time points and its close proximity with HIRA in infected cells, it is tempting to speculate that HBC may be required by HBV to evade host cell immune defense mechanisms before the initial chromatinization of the viral genome and might participate in the building of the cccDNA nucleosomal structure together with H3.3 deposited by the HIRA complex. Whether Hbc is required for HIRA recruitment onto incoming HBV genome remains to be investigated. Thanks to its C-terminal arginine-rich domain, Hbc could be assimilated to protamine-like protein VII of adenoviruses, but instead of being replaced by host cell histones on the viral genome, Hbc would integrate into the HBV chromatin structure to regulate nucleosomal spacing.

Altogether, our results indicate that the HIRA complex is a crucial proviral factor for HBV infection, determining the formation of its stable viral minichromosome and sustaining its transcriptional activity by promoting H3.3 deposition and recycling (Figure 13). Detection of H3.3S31ph bound to active cccDNA molecules opens exciting perspectives for the investigation of the role of the host chromatin regulatory machinery in determining the fate of HBV infection.

Methods

Production of WT and Mutated HBV Viral Inoculum

HBV (genotype D, subtype ayw) inoculum was prepared from filtered HepAD38 (WT HBV) or HepG2-H1.3-K6 cells (ΔHBx-HBV) supernatants by polyethylene-glycol-MW-8000 (PEG8000; Sigma) precipitation (8% final) as previously described. Viral stock with a titer reaching at least 8000 (PEG8000; Sigma) precipitation (8% final) as previously described. Viral stock with a titer reaching at least 8000 (PEG8000; Sigma) precipitation (8% final) as previously described. Viral stock with a titer reaching at least 8000 (PEG8000; Sigma) precipitation (8% final) as previously described.
HIRA-Mediated H3.3 Deposition on HBV cccDNA

1 × 10^{10} viral genome equivalents/mL was tested endotoxin free and used for infection. HepG2-H1.3-K6 cells were established by stable integration of a 1.3-fold HBV genome (genotype D, subtype ayw) carrying premature stop codon mutations at amino acid position 7 after the ATG in both the 5′ and 3′ HBX open reading frames, without affecting the other viral open reading frames (S, precore, core, and polymerase).

**PHH Isolation**

PHHs were isolated from surgical liver resections, after informed consent of patients (institutional review board agreements DC-2008-99 and DC-2008-101) as previously described and plated in complete William's medium supplemented with 1% penicillin/streptomycin (Life Technologies), 1% glutamine (Life Technologies), 5 μg/mL human insulin (Sigma-Aldrich), 25 μg/mL hydrocortisone hemisuccinate UPJ0HN (SERB), and 5% fetal calf serum (FCS) (Fetalclone II; PERBIO). PHHs were maintained in William's medium supplemented with 1.8% DMSO (Sigma-Aldrich) and infected with HBV within 72 hours after plating.

**Cell Culture and HBV Infection**

HepG2hNTCP cells were seeded at 10^5 cells/cm² in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (Life Technologies), streptomycin (Life Technologies), sodium pyruvate (Life Technologies), and 5% FCS (Fetalclone II). Tetracycline was added to the medium for 72 hours to induce HBc protein expression. BrdU was purchased from Sigma (B9285) and used at a concentration of 20 μmol/L to treat HepG2hNTCP cells 24 hours before HBV infection. Myristoylated preS1-myr (Myrcludex-like) peptide (sequence: GTNLSVPNPLGFFPDHQLDPAGFRANSNNPDWFDPNPNKDHWEANKVQ, synthesized by GeneScript) was used at 100 nmol/L to prevent virus entry into the cells.48,49

**Analysis of Viral Parameters During Replication**

Total DNA was purified from infected cells using the MasterPure Complete DNA Purification Kit (Lucigen) and quantified using the TaqMan Gene Expression assay (ID: Pa03453406_s1). To increase the specificity of HBV cccDNA detection, qPCR was preceded by a nuclease digestion using

---

Figure 12. HIRA is required for recruitment of H3.3, RNAPII, and H3.3S31ph to established cccDNA. (A–I) cccDNA-ChIP analysis at 9 dpi in CTL vs HIRA-depleted and trans-complemented conditions. (WT or W799A D800A mutant constructs). cccDNA ChIP was performed with (A) antibodies against HIRA, (C) cellular RNAPII, (D) H3.3, and its (E) phosphorylated form H3.3S31ph, (F) H3pan, and (G) H3.1/2. (H and I) No antibody (NoAb) or anti-E2F antibody served as ChIP technical negative controls. Graphs represent the means ± SEM of at least 3 independent experiments.
**Figure 13. Schematic representation of HIRA involvement in cccDNA formation and transcriptional activity in infected hepatocytes.** Once entered in the cell, the HBV nucleocapsid is shuttled to the nuclear pore, where the viral genome, the naked, partially double-stranded rcDNA covalently attached to the HBV polymerase, is released in the nucleoplasm together with the HBV core protein (HBC). Host DNA repair cellular machinery components, comprising tyrosyl-DNA phosphodiesterase-2 (TDP2), flap structure-specific endonuclease 1 (FEN-1), replication factor C (RFC), proliferating cell nuclear antigen (PCNA), translesion DNA polymerases, topoisomerases, and components of the ATR-CHK1 pathway are heavily involved in the biological reactions leading to viral polymerase and RNA primer eviction from rcDNA, as well as in viral DNA strand completion and ligation. The HIRA complex, through de novo H3.3 deposition, first ensures the building of HBV genome chromatin structure, allowing the establishment of the cccDNA pool, and then contributes to cccDNA active transcription, which is associated to H3.3 phosphorylation on S31.

---

**Cytotoxicity Assays**

Neutral red uptake assay and sulforhodamine staining were performed to assess cell viability after siRNA transfection and HIRA overexpression. For the neutral red uptake assay, the protocol was adapted from Repetto et al. After neutral red uptake assay, cells were washed 3 times with deionized water, dried, and processed for sulforhodamine staining.

**Plasmid and siRNA Cell Transfection**

pEYFP-N1-HIRA and pEYFP-N1-HIRA W799A D800A constructs were kindly provided by Ray-Gallet et al. and were transfected in HepG2-NTCP cells and PHH using TransIT-2020 (Mirus Bio, LLC) following the manufacturer’s protocol. siRNA targeting HIRA (ON-TARGETplus SMARTPool L-014117-01; Horizon Discovery) and SMC6-61 were transfected at 10 nmol/L concentration in HepG2NTCP cells and PHH using Lipofectamine RNAiMAX reagent (ThermoFisher), following the manufacturer’s instructions. siRNA targeting human SMC5 (ON-TARGETplus SMARTPool L-014117-01; Horizon Discovery) and SMC6-61 were transfected at 10 nmol/L concentration in HepG2NTCP cells and PHH using Lipofectamine RNAiMAX reagent (ThermoFisher), in parallel with ON-TARGETplus non-targeting pool (D-001810-10; Horizon Discovery) as negative control.
| Target             | Sequence                                                                 |
|--------------------|--------------------------------------------------------------------------|
| cccDNA_for         | CCGTGTGCACCTCGCTTCA                                                      |
| cccDNA_rev         | GCACAGCTTGGAGGCTTTGA                                                    |
| cccDNA_probe       | [6FAM]CATGGAGACCCAGCTCGAGAGG[BBQ]                                       |
| 3.5-kb RNA_for     | GAAGGACAAGCTCGCTTAAA                                                   |
| 3.5-kb RNA_rev     | GGATTGGAGACAGATCCCTATG                                                 |
| 3.5-kb RNA_probe   | [6FAM]AGGCAGGTCCCCTAGAAGAAGACCTCC[BBQ]                                  |
| HIRA_for           | GGCCTCGGAAGGACTTCA                                                     |
| HIRA_rev           | AGACAGACACATGGGCTCTT                                                  |
| NTCP_for           | GAGGAGGAAAGCTGGCTATATA                                                 |
| NTCP_rev           | GGATTGGAGACAGATCCCTATG                                                 |
| EXO1_for           | TQAGGGAATATAAGGGACGAGTT                                                |
| EXO1_rev           | AGTTTTCAGCACAAGAATAGCC                                                 |
| TDP2_for           | TGGAGTTTGCCTCGGTGCA                                                    |
| TDP2_rev           | TGGTTTCAGTCGGCTCTT                                                    |
| FEN1_for           | ATGACATCAAGACTCTTTGGC                                                  |
| FEN1_rev           | GGCAGAACAGAAATCAGAGACT                                                |
| POLk_for           | TQAGGGAATATAAGGGACGAGTT                                                |
| POLk_rev           | CTGCACGGAACACCAATCTCC                                                 |
| SNAI1 promoter_for | GCCTCCGTGAAACACTGGAATA                                                 |
| SNAI1 promoter_rev | GCACATCACTGAGAGGAGAGAG                                                 |
| SOX9 promoter_for  | CAGGAGGAAAGACACCAACAC                                                 |
| SOX9 promoter_rev  | CATACGACCTGAGACGACT                                                   |
| ZNF268 promoter_for| CCTGTTGTACGCTGTCTCTT                                                  |
| ZNF268 promoter_rev| GAATAAAGCCTGCTTGGAGTT                                                |
| POLQ gene body_for| GCAGTCCCTACTGGAATG                                                    |
| POLQ gene body_rev| TGGAGTTGAGAAGACCCCTTC                                                 |
| STAT1_for          | GTGAGGAAAGACACTGCCAT                                                  |
| STAT1_rev          | ACTGGAACCTGCTCTCAAGAC                                                 |
| STAT2_for          | CCCCATGCAGACCCTCAT                                                  |
| STAT_rev           | GAGTCTACAGCGACGACTT                                                  |
| Sp1_for            | GGTGAGCCAGCTGAGTGG                                                    |
| Sp1_rev            | CCAGGAGTACTGCTGACGACTT                                                |
| PPAR_for           | AGCGAGAAGACTGGAGAGAGAGAGAGCAGCCCA                                    |
| PPARa_rev          | GGCCTGTTGAGGAGTCTTCC                                                  |
| FXR (NR1H4) for Fw | GTGAGGAGGAGTGAAGGGTCTT                                                |
| FXR (NR1H4) rev    | CCCTGTACATACATACAGCA                                                   |
| HNF4a_for          | TCTTACGATTGACGAGAGG                                                   |
| HNF4a_rev          | TCTTACGATTGACGAGAGG                                                   |
| HNF1a_Ex2_for      | CTCAACAGCTCCACTGTC                                                    |
| HNF1a_Ex3_rev      | GCTGTTACATACAGCTCCC                                                  |
| CREB_for           | GTGGTGTACGTGGAGAGG                                                    |
| CREB_rev           | GCATCCTACACTGCTGAGT                                                  |
| CEBP γ_for         | TCTGTAACAGCTGCTC                                                      |
| CEBP γ_rev         | ATGTGTTACGCTGCTGAGG                                                  |
| SMC6_Ex14_for      | AGCAACGGAACTGGAGAGAT                                                   |
| SMC6_Ex15_rev      | TCTCTACACAGCTGGAGG                                                   |

**Table 1. Primers and Probes Sequences Used for TaqMan and SYBRgreen qPCR**
Western Blot
Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (PIC) and phenyl-methylsulfonyl fluoride (PMSF). Proteins were migrated in 4%–20% mini-PROTEAN TGX stain-Free Precast Gel (Bio-Rad Laboratories) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). Blots were blocked for 1 hour with 5% milk in 1x Tris buffered saline (Sigma) and stained with primary antibody in blocking buffer overnight at 4°C. After primary antibody incubation, blots were washed 3 times with 1x Tris buffered saline with 0.1% Tween 20, stained with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature, and washed again 3 times with 1x Tris buffered saline with 0.1% Tween 20. Detection occurred using the Bio-Rad Western Blotting system and chemiluminescent detection (ECL) according to the manufacturer’s recommendation. The antibodies used are listed in Table 2.

Northern Blot
Purified RNA was denatured at 50°C for 1 hour with glyoxal reagent (Life Technologies) and then subjected to electrophoresis through 1x phosphate buffer in a 1.2% agarose gel and transferred to a positively charged nylon membrane (Amersham N+; GE). Membrane-bound RNA was hybridized overnight at 42°C to DIG-labeled HBV-specific probes (Table 3, forward probes 1–8). The membrane was washed twice in low-stringency wash buffer (1x SSC, 0.1% sodium dodecyl sulfate [SDS]) for 30 minutes at room temperature and twice in high-stringency wash buffer (0.1x SSC, 0.1% SDS) for 30 minutes at 65°C. Detection was performed using anti-DIG alkaline phosphatase (1:20,000 dilution) and CDP-Star reagent (Roche) according to the manufacturer’s recommendation and imaged using the ChemiDoc MP imaging system (Bio-Rad). 18S and 28S ribosomal RNA signal was used as loading and quality control.

Table 2. List of Antibodies Used in the Work

| Target | Company | Dilution |
|--------|---------|----------|
| Hbc for PLA and ChIP | InVitrogen SC2362651 | 4 μg |
| Hbc for IF and FACS | Invitrogen MA1-7607 | 1:500 |
| HIRA for PLA, ChIP, and WB | Abcam Ab206655 | 2.5 μg/1:500 |
| H3.3 for ChIP | Abcam Ab62642 | 5 μg |
| H3.3S31ph for ChIP | Abcam ab962628 | 5 μg |
| RNA polymerase II for ChIP | Diagenode C15200004 | 1 μg |
| BrdU for ChIP | BD Pharmingen 555627 | 5 μg |
| E2F for ChIP | Santa Cruz | 5 μg |
| β-actin for WB | Abcam Ab6276 | 1:10,000 |
| H3pan for ChIP | Diagenode C15410324 | 2 μg |
| H2A for ChIP | Diagenode C15410166 | 2 μg |
| H2B for ChIP | Diagenode C15410157 | 1 μg |
| H4 for ChIP | Diagenode C15410156 | 2 μg |
| H3.1/3.2 for ChIP | Active motif AB_2793710 | 4 μg |
| Hbc for WB | Dako B0586 | 1:500 |
| HBs for IF | Abcam ab8636 | 1:100 |
| Goat anti-mouse IgG (H+L) secondary antibody, HRP | Invitrogen 62-6520 | 1:20,000 |
| Goat anti-rabbit IgG (H+L) secondary antibody, HRP | Invitrogen 31460 | 1:10,000 |
| PML for IF | Abcam ab179466 | 1:500 |
| NSE4 for ChIP | Abcepta AP9909a | 4 μg |

FACS, _____; H+L, _____; HBs, hepatitis B surface; HRP, horseradish peroxidase; IF, _____; PML, promyelocytic leukemia; WB, _____.
Table 3. DIG-Labeled HBV DNA Probe Sequences

| Target | Name  | Sequence        |
|--------|-------|-----------------|
| HBV    | F1    | TAGCGCCTCATTTTGTGGGT |
|        | F2    | TAGGACCGCTCTGTTGTA  |
|        | R2    | CCGTCGGAAGGTGTTTACA |
|        | R3    | ATGTTGATTTTGCGCCAG |
|        | R4    | GTTTGCGTCAGAAAACGTT |
|        | F4    | TGGACCTTTTGCGCTTCTC |
|        | R5    | AGGAGACCTAAGGGCTCC  |
|        | F6    | TACTGCACTAGGAAAGCAGA |
|        | R6    | TGGGAATCACCACCTCGGAA |
|        | F8    | AGACGAGGCTTCACTGGCC |
|        | R8    | ACCGACAAATAAGGGCGCTA |

Table 4. DIG-Labeled Mitochondrial DNA Probe Sequences

| Target          | Name   | Sequence       |
|-----------------|--------|----------------|
| Mitochondrial DNA | Fw-huND1 | CTCCTACTAAGCTGCTTCGATTTTAAT |
|                 | Rw-huND1 | CATAGGGCTGTAGGTTGTCGATTA |
|                 | Fw-huND5 | ATTTTTATCTCAACACTGCGATT |
|                 | Rw-huND5 | GGGCAAGTTTGCGTCTGTA |
|                 | Fw-huTP6 | CATTTACCAACACCACCAACTATC |
|                 | Rw-huTP6 | CGAAGGCTTAACTCAGGGCTC |

Flow Cytometry Analysis

After trypsinization, cells were pelleted and washed in PBS 1× and then fixed in 4% formaldehyde (pH 7.4) in PBS 1× for 10 minutes at room temperature. After centrifugation, permeabilization was performed in PBS supplemented with 0.1% Triton X-100 and 3% BSA. Cells were collected and resuspended in PBS supplemented with 3% BSA to proceed with the immunostaining. The primary antibody (Table 2) was added directly into the PBS/BSA 3% solution for 60 minutes on ice. PBS supplemented with 0.1% Triton X-100 and 3% BSA was used to wash the cells 2 times before centrifugation and incubation with the fluorochrome-conjugated secondary antibody (Table 2) for 30 minutes on ice in the dark. After 2 washes in PBS supplemented with 0.1% Triton X-100 and 3% BSA, the cells were resuspended in ice-cold PBS, 3% BSA, and 1% sodium azide, and analyzed directly on the flow cytometer (FacsCalibur; BD Biosciences).

ChIP

ChIP experiments were performed at the indicated time points (from 30 minutes to 72 hours) postinfection as previously described. Briefly, after a 15-minute crosslinking step with 1% formaldehyde at 37°C, nuclei were extracted in lysis buffer (5 mmol/L PIPES, 85 mmol/L KCl, 0.5% NP-40, 1 mmol/L PMSF, and 1× PIC) and resuspended in sonication buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl pH 8, 1 mmol/L PMSF, and 1× PIC) after centrifugation. After sonication and a precleaving step, chromatin was subjected to overnight immunoprecipitation at 4°C using 2–5 µg of antibodies indicated in Table 2 or no antibody. Immune complexes were then incubated for 2 hours with protein G agarose beads at 4°C, washed, and eluted in 10 mmol/L Tris-HCl pH 8, 5 mmol/L EDTA, 50 mmol/L NaCl, 1% SDS, 50 µg proteinase K, and 1× PIC.
Immunoprecipitated DNA was extracted and quantified by qPCR using cccDNA-specific primers (Table 1). Samples were normalized to input DNA using the ΔCt method where ΔCt = Ct (input) – Ct (immunoprecipitated) and expressed as a percentage of the input after normalization over no antibody signal (Figure 7I and J). As an additional negative control, ChIP experiments were performed with the cccDNA unrelated protein E2F (Figure 7K and L).

Specificity of antibodies used to immunoprecipitate H3.3 vs H3.1/2 variants was confirmed by analyzing the differential enrichment on genomic target sites shown to be preferentially bound by either H3.3 (Snai1 and Sox9 promoters) or H3.1/2 (Znf286 promoter and Polg gene body) both in HepG2NTCP cells and human liver tissue (Figure 9A–F).

Sequential Chromatin Immunoprecipitation

Cells were processed as for ChIP experiments until overnight immunoprecipitation. Immune complexes then were incubated for 2 hours with protein G agarose beads at 4°C, washed, and eluted in 10 mmol/L dithiothreitol. Eluted samples then were re-exposed to overnight immunoprecipitation at 4°C using 2–5 μg of antibodies in Re-ChIP buffer (1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, and 20 mmol/L Tris-HCl pH 8) and further processed as per the classic ChIP protocol described earlier.

Minicircle HBV Production and Transfection in HepG2-NTCP Cells

The plasmid pMC-HBV containing the full-length 3182 bp genotype D strain ayw HBV sequence was inserted into a parental plasmid vector generated from pMC.CMV-MCS-SV40polyA with the deletion of CMV promoter and polyA sequences as described by Yan et al. The 39-nucleotide attR site insertion in pMC-HBV was located immediately before the start codon of the preS1 gene, corresponding to the attR site insertion in pMC-HBV was located immediately before the start codon of the preS1 gene, corresponding to the middle of the terminal protein domain and spacer region of the polymerase gene to minimize the disruption of the HBV genome. ZCY10P3S2T competent bacteria (System Bioscience) then were transformed with the pMC-HBV and a single colony amplified in Terrific Broth over 12 hours at 42°C under 200 rpm agitation. Amplification was performed by adding 2 volumes of LB medium overnight at 42°C under 200 rpm agitation. Arabinose induction was performed at 32°C for 4 hours by adding LB medium supplemented with 0.1% L-arabinose and NaOH to ensure a pH of 7. After centrifugation at 4500 rpm for 10 minutes at 4°C, plasmid DNA was extracted using the Nucleobond Xtra Maxi endonuclease free kit according to the manufacturer’s instructions (Macherey-Nagel) and digested by the NdeI (New England Biolabs) restriction enzyme for 2 hours at 37°C and by a plasmid-safe DNase (System Bioscience) overnight at 37°C to linearize the parental construct and get rid of it. After purification, the resulting plasmid was observed on agarose gel to check for the elimination of the parental plasmid. Eighty percent to 90% confluent HepG2NTCP cells were transfected with the pMC-HBV plasmids and the TransIT-2020 (Mirus) transfecting agent according to the manufacturer’s instructions in DMEM supplemented with 5% FCS, 1% GlutaMAX, and 1% sodium pyruvate. The following day, cells were washed once with 1× PBS and cultured for 3 more days in DMEM supplemented with 5% FCS, 1% GlutaMAX, 1% sodium pyruvate, and 1% penicillin-streptomycin.

Human Liver Samples

Human liver samples derived from 3 untreated chronic hepatitis B male patients belonging to a historical cohort collected at Hospices Civils de Lyon (Lyon University, Lyon, France). These patients underwent liver biopsy as part of their clinical follow-up evaluation, a fragment was preserved for research purposes and stored at -80°C. The protocol was approved by the competent Institutional Ethics Committee (CPP Sud est IV 11/040, authorization number DC-2008-235). Written informed consent was obtained from all patients and/or their legal guardians to undergo a liver biopsy. No patients were co-infected with human immunodeficiency virus, hepatitis C virus, or hepatitis delta virus.

Statistical Analysis

Statistics were performed using R (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). RT-qPCR and ChIP enrichment values obtained from the different drug treatments were compared using the 2/K sample permutation test with Monte Carlo resampling approximation and a 2-tailed P value was calculated for a risk threshold of .05.

All authors had access to the study data and reviewed and approved the final manuscript.

References

1. Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. Virology 2015;479–480C:672–686.
2. Lieberman PM. Chromatin regulation of virus infection. Trends Microbiol 2006;14:132–140.
3. Xia Y, Guo H. Hepatitis B virus cccDNA: formation, regulation and therapeutic potential. Antiviral Res 2020;180:104824.
4. Fanning GC, Zoulim F, Hou J, Bertolletti A. Therapeutic strategies for hepatitis B virus infection: towards a cure. Nat Rev Drug Discov 2019;18:827–844.
5. Saper G, Kler S, Asor R, Oppenheim A, Raviv U, Harries D. Effect of capsid confinement on the chromatin organization of the SV40 minichromosome. Nucleic Acids Res 2013;41:1569–1580.
6. Schreiner S, Nossal M. A role for the host DNA damage response in hepatitis B virus cccDNA formation and beyond? Viruses 2017;9:125.
7. Kitamura K, Que L, Shimada M, Koura M, Ishihara Y, Wakae K, Nakamura T, Watashi K, Wakita T, Muramatsu M. Flap endonuclease 1 is involved in cccDNA formation in the hepatitis B virus. PLoS Pathog 2018;14:e1007124.
8. Long Q, Yan R, Hu J, Cai D, Mitra B, Kim ES, Marchetti A, Zhang H, Wang S, Liu Y, Huang A, Guo H. The role of...
host DNA ligases in hepadnavirus covalently closed circular DNA formation. PLoS Pathog 2017;13:e1006784.

10. Wei L, Ploss A. Core components of DNA lagging strand synthesis machinery are essential for hepatitis B virus cccDNA formation. Nat Microbiol 2020;5:715–726.

11. Königer C, Wingert I, Marsmann M, Rösler C, Beck J, Nasal M. Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA (cccDNA) and persistence reservoir of hepatitis B viruses. Proc Natl Acad Sci U S A 2014;111:E4244–E4253.

16. Ray-Gallet D, Ricketts MD, Sato Y, Gupta K, Almouzni G, Nakatani Y. Histone H3.3 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell 2004;116:51–61.

17. Ray-Gallet D, Ricketts MD, Sato Y, Gupta K, Boyarchuk E, Sendai T, Marmorstein R, Almouzni G. Functional activity of the H3.3 histone chaperone complex HiRA requires trimethylation of the HIRA subunit. Nat Commun 2018;9:3103.

18. Rai TS, Glass M, Cole JJ, Rather ML, Marsden M, Neilson M, Brock C, Humphreys IR, Everett RD, Adams PD. Histone chaperone HIRA deposits histone H3.3 onto foreign viral DNA and contributes to anti-viral intracellular immunity. Nucleic Acids Res 2017;45:11673–11683.

19. Sitbon D, Boyarchuk E, Dingli F, Leow D, Almouzni G. Histone variant H3.3 residue S31 is essential for Xenopus gastrulation regardless of the deposition pathway. Nat Commun 2020;11:1256.

20. Martíre S, Gogate AA, Whitmill A, Tafessu A, Nguyen J, Teng Y-C, Tastemel M, Banaszynski LA. Phosphorylation of histone H3.3 at serine 31 promotes p300 activity and enhancer acetylation. Nat Genet 2019;51:941–946.
2713. Alvarez-Astudillo F, Garrido D, Varas-Godoy M, Gutiérrez JL, Villanueva RA, Loyola A. The histone variant H3.3 regulates the transcription of the hepatitis B virus. Ann Hepatol 2021;21:100261.  
2714. Allweiss L, Giersch K, Pirosu A, Volz T, Muench RC, Beran RK, Urban S, Javanbakht H, Fletcher SP, Lütgheitmann M, Dandi M. Therapeutic shutdown of HBV transcripts promotes reappearance of the SMCC5/6 complex and silencing of the viral genome in vivo. Gut 2022;71:372–381.  
2715. Ko C, Chakraborty A, Chou W-M, Hasreiter J, Wettengel JM, Stadler D, Bester R, Asen T, Zhang K, Wisskirchen K, McKeating JA, Ryu W-S, Protzer U. Hepatitis B virus genome recycling and de novo secondary infection events maintain stable cccDNA levels. J Hepatol 2018;69:1231–1241.  
2716. Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, Zentgraf H. Structural organization of the hepatitis B virus minichromosome. J Mol Biol 2001;307:183–196.  
2717. Tropberger P, Mercier A, Robinson M, Zhong W, Adam S, Polo SE, Almouzni G. Transcription recovery after DNA damage requires chromatin priming by the H3.3 histone chaperone HIRA. Cell 2013;155:94–106.  
2718. Yang G, Feng J, Liu Y, Zhao M, Yuan Y, Yuan H, Yun H, Sun M, Bu Y, Liu L, Liu Z, Niu J-Q, Yin M, Song X, Miao Z, Lin Z, Zhang X. HAT1 signaling confers to assembly and epigenetic regulation of HBV cccDNA minichromosome. Theranostics 2019;9:7345–7358.  
2719. Cohen C, Corpet A, Roubille S, Marouei M, Rousseau A, Kleijwegt C, Bind A, Texier P, Sawtell N, Labetoulle M, Lomonte P. Promyelocytic leukemia (PML) nuclear bodies (NBs) induce latent/quiescent HSV-1 genomes chromatinization through a PML NB/histone H3.3/H3.3 chaperone axis. PLoS Pathog 2018;14:e1007313.  
2720. Niu C, Livingston CM, Li L, Beran RK, Dafnif1, Niu C, Livingston CM, Li L, Peiser L, Salas E, Wang GH, Neveu G, Alam A, Fraisse L, Carter K, Testoni B, Zoulim F. Full-length 5’RACE identifies all major HBV transcripts in HBV-infected hepatocytes and patient serum. J Hepatol 2020;73:40–51.  
2721. Ni Y, Lempp FA, Mehrle S, Nkongolo S, Kaufman C, Falth M, Stindt J, König H, Nessel M, Kubitz R, Siltmann H, Urban S. Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology 2014;146:1070–1083.  
2722. Allweiss L, Volz T, Giersch K, Kaj H, Raffa G, Petersen J, Lok HWE, Beninati C, Policino T, Urban S, Lütgheitmann M, Dandi M. Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. Gut 2018;67:542–552.  
2723. Leboussé F, Inchauspé A, Locatelli M, Maiglia C, Diederichs A, Fresquet J, Chaupe F, Hamed K, Testoni B, Zoulim F. Quantification and epigenetic evaluation of the residual pool of hepatitis B covalently closed circular DNA in long-term nucleoside analogue-treated patients. Sci Rep 2020;10:21097.  
2724. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 2008;3:1125–1131.  
2725. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006;1:1112–1116.  
2726. Gomes AP, Iltor D, Low V, Rosenzweig A, Shen Z-J, Schild T, Rivas MA, Er EE, McNally DR, Mutvei AP, Han J, Ou Y-H, Cavaliere P, Mullarky E, Nagiec M, Shin S, Yoon S-O, Dephoure N, Massagué J, Melnick AM, Cantley LC, Tyler JK, Blenis J. Dynamic incorporation of histone H3 variants into chromatin is essential for acquisition of aggressive traits and metastatic colonization. Cancer Cell 2019;36:402–417.e13.
Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by Labex DEVweCAN (ANR-10-LABX-61) and by the French National Agency for Research on human immunodeficiency virus and viral hepatitis (ANRS, grant ECTZ93319) (F.Z. and B.T.) and for the GA team by la Ligue Nationale Contre le Cancer (Equipe Labellisée Ligue), Labex DEEP (ANR-11-LABX-0044, DEEP, ANR-10-IDEX-0001-02), PSL, and ERC-2015-ADG-694694 ChromADICT.

Data Transparency Statement
The authors declare that all the data supporting the findings of this study are available within the article or from the corresponding authors upon reasonable request.