Short communication

JHEP|Reports

Evidence for long-term association of virion-delivered HBV core protein with cccDNA independently of viral protein production

Julie Lucifora,[1,2,*,†] Florentin Pastor,[1,†] Émilie Charles,[1] Caroline Pons,[1,2] Héloïse Auclair,[2] Floriane Fusil,[1] Michel Rivoire,[3] François-Loïc Cosset,[1] David Durantel,[1,*,‡] Anna Salvetti[1,*,‡]

[1]*CIRI – Centre International de Recherche en Infectiologie, Univ Lyon, Université Claude Bernard Lyon 1, Inserm, U1111, CNRS, UMR5308, ENS Lyon, F-69007 Lyon, France;* [2]*Past affiliation: INSERM, U1052, Cancer Research Center of Lyon (CRCL), University of Lyon (UCBL1), CNRS UMR_5286, Centre Léon Bérard, Lyon, France;* [3]*INSERM U1032, Centre Léon Bérard (CLB), Lyon, France*

JHEP Reports **2021.** https://doi.org/10.1016/j.jhepr.2021.100330

Background & Aims: HBV persists in the nucleus of infected hepatocytes as a covalently closed circular DNA (cccDNA) episome that constitutes the template for viral RNA and protein synthesis. Both HBx and HBc (core) viral proteins associate with cccDNA but, while HBx is required for viral transcription, the role of HBc is still unclear. The aim of this study was to determine if HBc derived from incoming nucleocapsid can associate with cccDNA before the onset of viral transcription and protein production.

Methods: Chromatin immunoprecipitation assays were performed in native conditions. In addition, differentiated HepaRG (dHepaRG) cells infected with HBx-deficient HBV were used to investigate if HBc delivered by incoming virions can associate with cccDNA.

Results: Our results indicate that HBc can associate with cccDNA in the absence of viral transcription and *de novo* protein synthesis. In dHepaRG cells, this association is stable for at least 6 weeks.

Conclusion: These results suggest that virion-delivered HBc may participate at an early stage of cccDNA formation and/or transcription.

Lay summary: The hepatitis B virus genome is released into the nucleoplasm of infected cells after disassembly of the viral nucleocapsids at the nuclear membrane. Herein, we show for the first time that virion-delivered hepatitis B core protein, a component of the viral capsid, can stably associate with integrated viral DNA.

© 2021 The Authors. Published by Elsevier B.V. on behalf of European Association for the Study of the Liver (EASL). This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

HBV functionally persists in the nucleus of hepatocytes as a DNA episome (covalently closed circular DNA [cccDNA]) that is organized into a chromatin-like structure to which host and viral proteins associate to regulate its transcription, as shown by chromatin immunoprecipitation (ChIP) analyses.[1,2] Among the viral proteins, HBx associates with cccDNA and is essential for HBV RNA synthesis.[3,4] HBV core protein (HBc), the structural component of the capsid, can also associate with cccDNA and is believed to contribute to its structure and/or transcriptional activit.[5,6] The origin of cccDNA-associated HBc is currently unclear. In particular, it is not known whether HBc derived from incoming capsids – delivered to the nucleus together with relaxed circular DNA (rcDNA) – can associate with cccDNA. Herein, we developed a sensitive ChIP method to efficiently detect the association of virion-delivered HBc to cccDNA in human primary hepatocytes *in vitro* and *in vivo*. Using this procedure, we show that HBc derived from incoming capsids can associate with cccDNA in the absence of *de novo* synthesis of viral proteins.

Materials and methods

In vitro/*in vivo* HBV infections and virologic analyses

HepaRG and derived cell lines, primary human hepatocytes (PHHs) and liver-humanized FRG mice were maintained and infected with HBV as previously described.[7,8] PHHs were isolated from hepatic resections obtained in collaboration with surgical departments of Lyon (Centre Léon Bérard) with the French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011). All experiments with liver-humanized mice (HuHeps) were performed in accordance with the European Union guidelines for approval of the protocols by the local ethics committee (Authorization Agreement C2EA-15, "Comité Rhône-Alpes d'Ethique pour l'Expérimentation Animale", Lyon, France - APAFIS#1570-2015073112163780). Five 20-week-old humanized female FRG (9 weeks post engraftment of PHHs) mice were used. HBeAg was quantified by CLIA according to manufacturer's instructions (AutoBio, China). Hirt extractions

Keywords: Hepatitis B Virus; HBc; cccDNA; ChIP; CAM.
Received 19 January 2021; received in revised form 2 June 2021; accepted 21 June 2021; available online 8 July 2021

† Contributed equally as first authors

‡ Contributed equally as senior authors

* Corresponding authors: Address: CIRI – Centre International de Recherche en Infectiologie, Univ Lyon, Université Claude Bernard Lyon 1, Inserm, U1111, CNRS, UMR5308, ENS Lyon, F-69007 Lyon, France.
E-mail addresses: julie.lucifora@inserm.fr (J. Lucifora), or david.durantel@inserm.fr (D. Durantel), or anna.salvetti@inserm.fr (A. Salvetti).

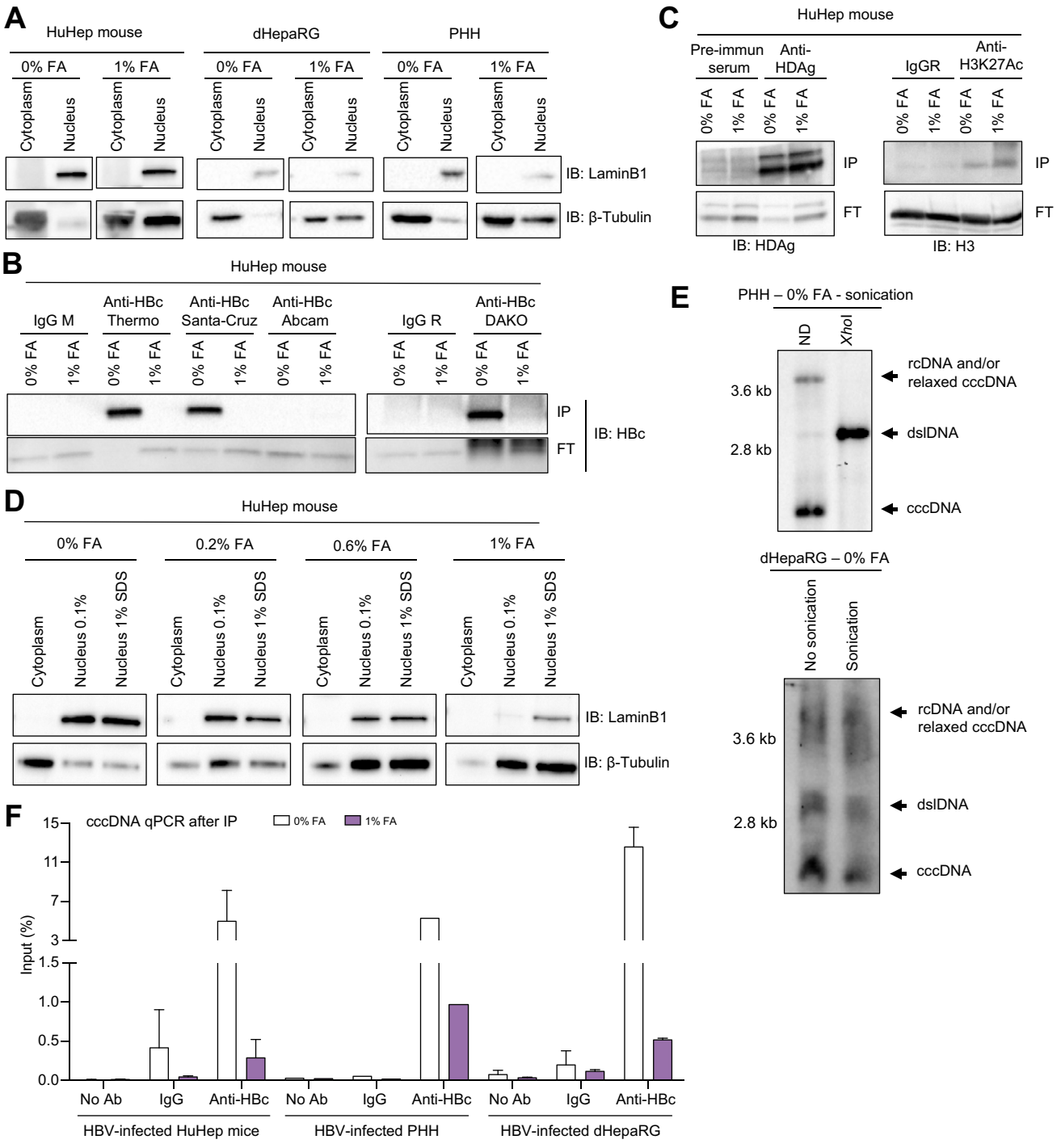

Fig. 1. Cross-linking of samples prevents good nuclear purification and efficient anti-HBc IP. (A) Cells or liver samples from HuHep mice were cross-linked or not with FA before nuclear fractionation and analyses by WB. (B, C) Liver samples from (B) HBV-infected or (C) HBV/HDV co-infected HuHep mice were either cross-linked with 1 % FA or left untreated (0 % FA) before nuclei isolation and IP with indicated antibodies. IP efficiencies were analyzed by WB. (D) A liver sample from a HuHep mouse was divided into 4 pieces before cross-linking with the indicated amount of FA. Cytoplasmic and nuclear extracts were prepared with different concentration of SDS for nuclear lysis and analyzed by WB. (E) PHHs or dHepaRG cells were infected by HBV (500 vge/cell). Ten days later, nuclear extracts were prepared in native conditions, sonicated or not and HBV DNA was analyzed by Southern Blot. (F) dHepaRG cells or PHHs were infected with HBV wild-type for 10 days. HuHep mice were infected with HBV for 7 weeks. Half of each sample was either cross-linked with 1% FA or left untreated before nuclei isolation and IP with the indicated antibodies from Thermofisher followed by DNA extraction. The % of HBc-associated cccDNA, relative to the amount in the input, was assessed by qPCR. Results are the mean ± SD of 2 experiments for dHepaRG cells and HuHep mice and 1 experiment for PHHs. cccDNA, covalently closed circular DNA; dHepaRG, differentiated HepaRG cells; dslDNA, double-stranded linear DNA; FA, formaldehyde; FT, flow through; IP, immunoprecipitation; HBc, HBV core protein; HuHep, liver-humanized mice; PHHs, primary human hepatocytes; rcDNA, relaxed circular DNA; vge, viral genome equivalents.

and Southern blot for the detection of HBV replication intermediates, as well as PCR and reverse-transcription quantitative PCR (RT-qPCR) for cccDNA and total HBV RNA quantification were performed as previously described.[8]

Chromatin immunoprecipitation assays

Frozen cells or liver pieces were chemically cross-linked with 1% formaldehyde (FA) for 10 min at RT, quenched with 0.25 M glycine for 5 min, and mechanically lysed at 4°C in lysis buffer (PIPES 5 mM, KCL 85 mM, NP-40 0.5%), with protease inhibitors (Sigma-Aldrich). After low-speed centrifugation, nuclei were lysed in SDS-buffer (EDTA 10 mM, Tris-HCl pH8 50 mM, SDS 1%). After sonication, clarified lysates were diluted in RIPA-buffer (Tris-HCl pH-7.5 10 mM, NaCl 140 mM, EDTA 1 mM, EGTA 0.5 mM, 1% Triton-X100, 0.1% SDS, 0.1% Na-Deoxycholate). Native samples were treated as above except that nuclei were lysed in RIPA-buffer before sonication. Nuclear lysates were precleared with magnetic beads (Dynabeads™ Protein G, Thermo-Fisher) before immunoprecipitation with antibodies at 4°C. Magnetic beads were added for 2 h to capture immune complexes. Flow-through fractions were collected, and beads washed with RIPA-buffer. Immunoprecipitated complexes were either boiled in Laemmli 1X for western blot analysis or washed with TE (Tris-HCl pH-8 10 mM, EDTA 10 mM) and then incubated for 2 h at 68°C in elution buffer (Tris-HCl pH-7.5 20 mM; EDTA 5 mM; NaCl 50 mM; 1% SDS; Proteinase-K 50 μg/ml). DNA was then extracted and quantified by qPCR.[7] DNA was similarly purified from the flow-through fraction and used as input for calculations. We used the following antibodies for immunoprecipitation: anti-mouse IgG (IgG-M) (Thermo-Fisher, MA5-14447), anti-HBc (Thermo-Fisher, MA1-7607), anti-HBc (Abcam, ab8638), HBc (Santa-Cruz, sc-23945), anti-HBc (DAKO, B0586), rabbit IgG (IgG-R) (Cell Signaling, cs2729), anti-H3K27Ac (Diagenode, MAb-184-050), polyclonal rabbit anti-HDAg antibody (a kind gift from Janssen). We used the following antibodies for western blot: anti-β-Tubulin (Abcam, Ab6046), anti-Lamin B1 (Abcam, Ab16048), anti-H3 (Abcam, Ab1791), polyclonal rabbit anti-HBc antibody (a kind gift from Dr Adam Zlotnick). Full blots are shown at the end of the supplementary information file.

Results

The analysis of the specific association of HBc to cccDNA by ChIP involves 2 critical steps. First, nuclei should be efficiently isolated from the cytoplasmic fraction to optimize the ratio of cccDNA to rcDNA and allow its selective quantification by PCR. Second,

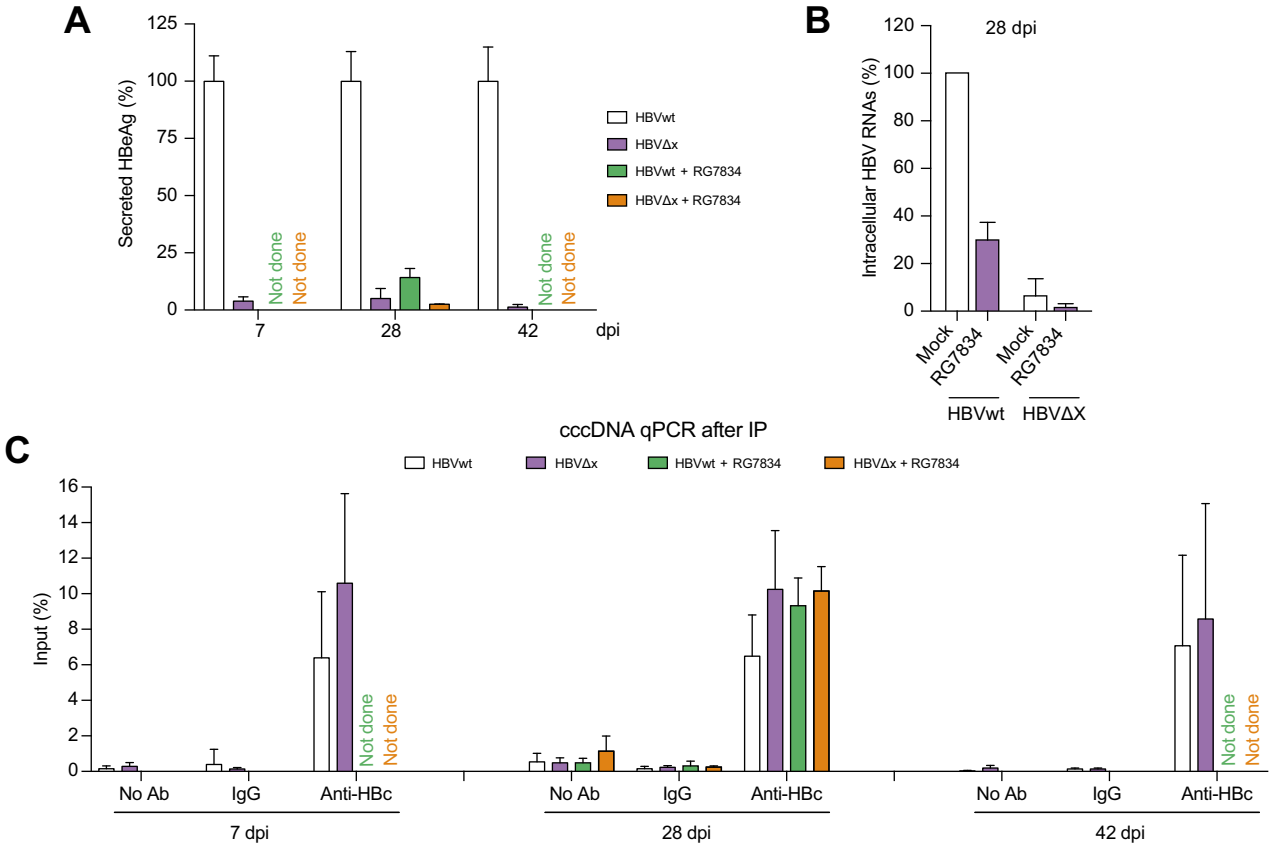

Fig. 2. HBc derived from incoming HBV capsids associates with cccDNA in a stable manner. (A, B, C) dHepaRG cells were infected with HBV WT or HBVΔX (1,000 vge/cell). Starting at day 7 pi, cells were either treated twice a week with RG7834 (0.1 uM) or left untreated. At the indicated time, (A) supernatants were collected and levels of HBeAg assessed by ELISA. (B) At day 28, RNAs were extracted and the levels of HBV RNAs assessed by RT-qPCR analyses. Native ChIP assays were performed at the indicated time and % of HBc associated to cccDNA assessed by qPCR analysis. Results are the mean ± SD of 2 to 5 independent experiments each performed with 2 biological replicates (2 experiments for 42 dpi, 3 experiments for cells treated with RG7834, 5 for the other conditions). cccDNA, covalently closed circular DNA; ChIP, chromatin immunoprecipitation; dHepaRG, differentiated HepaRG cells; HBc, HBV core protein; HBVΔX, HBx-deficient HBV; RT-qPCR, reverse-transcription PCR; vge, viral genome equivalents; WT, wild-type.

immunoprecipitation of HBc/cccDNA complexes should be very efficient. In order to stabilize the interactions between DNA and proteins, ChIP is usually performed on 1% FA-cross-linked samples. However, using this method we did not obtain a satisfactory purification of nuclei from either HuHep mice livers, dHepaRG cells or PHHs. In contrast, an improved cell fractionation was achieved using native samples (0% FA) (Fig. 1A). In addition, we observed that several anti-HBc antibodies currently used for ChIP analyses showed a very good immunoprecipitation efficiency with native but not cross-linked samples (Fig. 1B). This was not observed with other antibodies, such as anti-H3K27Ac or anti-HDAg antibodies, which showed equal immunoprecipitation efficiencies in both conditions (Fig. 1C). The need for high concentrations of SDS to efficiently lyse nuclei extracted from 1% FA-cross-linked cells rather than the cross-linking itself may explain why immunoprecipitation of anti-HBc was not efficient (Fig. 1D). Importantly, supercoiled cccDNA was still detectable after sonication of nuclear extracts (in the native condition as well as after cross-linking) at levels (compared to rcDNA) allowing its specific detection by qPCR (Fig. 1E, S1). Importantly, recovery of cccDNA after immunoprecipitation of HBc was much higher in native samples from HBV-infected HuHep mice livers, dHepaRG cells or PHHs compared to samples cross-linked with 1% FA (Fig. 1F). Even if nuclei purification and, consequently, discrimination between cccDNA and rcDNA was not as good as that obtained with 0% FA, mild cross-linking (*i.e.* using 0.2%–0.6% FA) that enables nuclei lysis with low SDS concentrations can also be used for anti-HBc ChIP (Fig. S2).

Having established this improved ChIP procedure, we asked if cccDNA-associated HBc could be detected in the absence of viral transcription and, therefore, of *de novo* protein synthesis. The HBVΔX virus can establish cccDNA after infection of hepatocytes but cannot support the synthesis of viral RNAs and, consequently, of viral proteins such as HBc or HBeAg[3] (Fig. S3, Fig. 2A,B). Therefore, the HBc protein present in HBVΔX-infected cells can derive only from incoming capsids. Interestingly, cccDNA-specific-ChIP assays performed in native conditions revealed an HBc signal associated with cccDNA in HBVΔX-infected dHepaRG cells that lasted for as long as that observed in cells infected with wild-type HBV. To rule out any possible contamination with newly synthesized HBc in the HBVΔX-infected cells, cells were treated with the HBV RNA destabilizer RG7834 (Fig. 2B).[9] In this condition, no difference in the HBc signal associated with cccDNA was observed compared to mock-treated cells (Fig. 2C). HBc signals were also detected using mildly cross-linked samples, strongly suggesting that the association of HBc to cccDNA in native conditions was not due to artificial binding during immunoprecipitation (Fig. S4).

Discussion

Besides its role in the cytoplasm, the HBc protein is present in the nucleus where it is believed to play important regulatory functions by associating with cccDNA. Two studies performed in hepatocytes infected with mutant HBV virions incapable of producing HBc showed that the newly synthetized protein was not required for the maintenance and transcriptional activity of cccDNA.[10,11] However, these studies did not examine whether HBc derived from incoming virions was associated with cccDNA, leaving open the possibility that, by doing so, it could play a role during cccDNA establishment and/or transcription.

Our results indicate that, in dHepaRG cells, HBc derived from incoming HBV capsids can associate with cccDNA without the need for viral transcription and *de novo* protein synthesis. Whether this association occurs immediately with rcDNA, as soon as it is released into the nucleoplasm or after its conversion on cccDNA is presently unknown. An attractive hypothesis is that by associating to rcDNA, input HBc may participate in its conversion into cccDNA. We observed an extremely stable association of HBc derived from incoming HBV capsids with transcriptionally inactive cccDNA (with HBVΔX). It remains to be investigated if active viral transcription results in a dynamic replacement of virion-derived HBc with newly synthetized proteins. These questions are of particular relevance as several drugs targeting HBc (capsid assembly modulators) are being clinically evaluated and will likely become the next approved class of anti-HBV antivirals.[12,13]

Abbreviations

cccDNA, covalently closed circular DNA; ChIP, chromatin immunoprecipitation; dHepaRG, differentiated HepaRG cells; FA, formaldehyde; H3K27Ac, histone 3 lysine 27 acetylation; HBc, HBV core protein; HBVΔX, HBx-deficient HBV; HuHep, liver-humanized mice; PHHs, primary human hepatocytes; rcDNA, relaxed circular DNA.

Financial support

This work was funded by the « Institut National de la Santé et de la Recherche Médicale » (INSERM), « Centre National de la Recherche Scientifique » (CNRS), and « Université Claude Bernard Lyon 1 » (UCBL). It was also supported by grants from ANRS (« Agence Nationale de Recherche sur le Sida et les hépatites virales »; several grants from « CSS12 ») and by the LabEx Ecofect (ANR-11-LABX-0048) of the "Université de Lyon", within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JL, AS, DD: Study concept and design; JL, FP, EC, HA, CP, FF: acquisition of data; JL, AS, DD: writing of the manuscript; MR, FLC: material support.

Data availability statement

The data shown in this article are available from the corresponding authors upon a reasonable request. All reagents, antibodies and resources used in this research can be found in the CTAT table.

Acknowledgements

The authors would like to thank Dr Barbara Testoni and Dr Christine Neveut for advice on ChIP assays, Dr Frederik Pauwels (Janssen) for the kind gift of anti-HDAg antibodies. We would also like to thank Maud Michelet, Jennifer Molle and Anaëlle Dubois for precious help with the isolation of primary human hepatocytes, as well as the staff of Prof Michel Rivoire for providing us with liver resections.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2021.100330.

References

Author names in bold designate shared co-first authorship

[1] Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015;64:1972–1984.

[2] Lucifora J, Protzer U. Attacking hepatitis B virus cccDNA–The holy grail to hepatitis B cure. J Hepatol 2016;64:S41–S48.

[3] **Lucifora J**, **Arzberger S**, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol 2011;55:996–1003.

[4] **Decorsiere A**, **Mueller H**, **van Breugel PC**, Abdul F, Gerossier L, Beran RK, et al. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. Nature 2016;531:386–389.

[5] Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, et al. Structural organization of the hepatitis B virus minichromosome. J Mol Biol 2001;307:183–196.

[6] Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, et al. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. Gastroenterology 2006;130:823–837.

[7] **Lucifora J**, **Bonnin M**, Aillot L, Fusil F, Maadadi S, Dimier L, et al. Direct antiviral properties of TLR ligands against HBV replication in immune-competent hepatocytes. Scientific Rep 2018;8:5390.

[8] Lucifora J, Salvetti A, Marniquet X, Mailly L, Testoni B, Fusil F, et al. Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector. Antivir Res 2017;145:14–19.

[9] **Mueller H**, **Lopez A**, Tropberger P, Wildum S, Schmaler J, Pedersen L, et al. PAPD5/7 are host factors that are required for hepatitis B virus RNA stabilization. Hepatology 2018.

[10] Qi Y, Gao Z, Xu G, Peng B, Liu C, Yan H, et al. DNA polymerase kappa is a key cellular factor for the formation of covalently closed circular DNA of hepatitis B virus. Plos Pathog 2016;12:e1005893.

[11] **Tu T**, **Zehnder B**, Qu B, Urban S. D e novo synthesis of hepatitis B virus nucleocapsids is dispensable for the maintenance and transcriptional regulation of cccDNA. JHEP Rep 2021;3:100195.

[12] Xia Y, Liang TJ. Development of direct-acting antiviral and host-targeting agents for treatment of hepatitis B virus infection. Gastroenterology 2019;156:311–324.

[13] Fanning GC, Zoulim F, Hou J, Bertoletti A. Therapeutic strategies for hepatitis B virus infection: towards a cure. Nat Rev Drug Discov 2019;18:827–844.