

Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector

Julie Lucifora, Anna Salvetti, Xavier Marniquet, Laurent Mailly, Barbara Testoni, Floriane Fusil, Aurore Inchauspé, Maud Michelet, Marie-Louise

Michel, Massimo Levrero, et al.

▶ To cite this version:

Julie Lucifora, Anna Salvetti, Xavier Marniquet, Laurent Mailly, Barbara Testoni, et al.. Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector. Antiviral Research, 2017, 145, pp.14-19. 10.1016/j.antiviral.2017.07.006 . hal-01953660

HAL Id: hal-01953660 https://hal.science/hal-01953660

Submitted on 23 Aug 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector

Julie Lucifora¹, Anna Salvetti¹, Xavier Marniquet², Laurent Mailly³, Barbara Testoni¹, Floriane Fusil⁴, Aurore Inchauspé^{1,2}, Maud Michelet¹, Marie-Louise Michel⁵, Massimo Levrero¹, Pierre Cortez², Thomas F. Baumert^{3,6}, François-Loic Cosset⁴, Cécile Challier², Fabien Zoulim^{1,7,8,#} and David Durantel^{1,#}

¹INSERM, U1052, Cancer Research Center of Lyon (CRCL), Université de Lyon (UCBL1), CNRS UMR_5286, Centre Léon Bérard, Lyon, France ;

²Sanofi R&D, Infectious Disease Therapeutic Area, Marcy l'Etoile, France ;

³INSERM U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, Strasbourg, France ;

⁴INSERM, U1111, International Center for Infectiology Research (CIRI), Team EVIR, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, Ecole Normale Supérieure de Lyon, Univ Lyon, F-69007, Lyon, France

⁵INSERM U994, Institut Pasteur, Paris, France

⁶Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

⁷Hospices Civils de Lyon (HCL), Lyon, France

⁸Institut Universitaire de France (IUF), Paris, France

#Corresponding authors

Dr. David Durantel and Prof. Fabien Zoulim

Cancer Research Center of Lyon (CRCL), UMR INSERM U1052 - CNRS 5286, 151 cours Albert Thomas, 69424 Lyon Cedex 03, France ; Phone: + 33 4 72 68 19 70 ; Fax : +33 4 72 68 19 71 ; E-mail : <u>david.durantel@inserm.fr</u> ; <u>fabien.zoulim@inserm.fr</u>

Abbreviations

AAV: adeno-associated virus, cccDNA: covalently closed circular DNA, DIG: digoxigenin, dsl: double stranded linear DNA, HBV: Hepatitis B Virus, HDV: Hepatitis Delta Virus, kb: kilo base, MW: molecular weight, NTCP: sodium taurocholate cotransporting polypeptide, p.i.: post-infection, p.t.: post-transduction, qPCR: quantitative PCR, rcDNA: relaxed circular DNA, ss: single stranded DNA.



Highlights

- The HBV cccDNA was detected by Southern blotting in AAV-HBV transduced C57BL6 mice
- A strategy was set up to distinguish the HBV cccDNA from the AAV-HBV episome by qPCR
- AAV-HBV transduced immune-competent mice will allow to test drugs targeting cccDNA regulation and stability *in vivo*

Abstract

Hepatitis B Virus (HBV) persists in infected hepatocytes as an episomal covalently-closedcircular DNA mini-chromosome, called cccDNA. As the main nuclear transcription template, HBV cccDNA is a key replication intermediate in the viral life cycle. Little is known about the mechanisms involved in its formation, maintenance and fate under antiviral therapies. This is mainly due to the lack of small immune-competent animal models able to recapitulate the entire HBV replication cycle, including formation of HBV cccDNA. Here we report that HBV cccDNA can be detected by Southern blot analyses in the liver of C57BL6 mice transduced with AAV-HBV. HBV cccDNA persists in the liver of these animals together with the AAV-HBV episome. We also set up a PCR strategy to distinguish the HBV cccDNA from the AAV-HBV episome. These suggest that the AAV-HBV/mouse model might be relevant to test drugs targeting HBV cccDNA regulation and persistence.

Keywords

Hepatitis B virus, cccDNA, adeno-associated virus, immune-competent mouse, immunetherapeutics

Highlights

- The HBV cccDNA was detected by Southern blotting in AAV-HBV transduced C57BL6 mice
- A strategy was set up to distinguish the HBV cccDNA from the AAV-HBV episome by qPCR
- AAV-HBV transduced immune-competent mice will allow to test drugs targeting cccDNA regulation and stability *in vivo*

Main text

The Hepatitis B virus (HBV) circulates in the blood of patients as virions containing a partially double stranded relaxed circular DNA (rcDNA). However, the virus persists in the nucleus of infected hepatocytes as a covalently-closed-circular DNA, called HBV cccDNA, which is the template for all viral RNAs production. HBV cccDNA is organized in a chromatin-like structure that displays a typical beads-on-a-string arrangement by electron microscopy [1, 2]. Host histone proteins as well as other proteins involved in gene expressing regulation are bound to HBV cccDNA [3] and its transcriptional activity is subjected to the "histone code" [4]. Since it does not contain an origin of replication, HBV cccDNA persistence relies on its stability in non-dividing cells and/or on its replenishment via re-import of rcDNA from neo-synthesized nucleocapsids [5]. The universal rebound of HBV replication upon withdrawal from nucleos(t)ide analogue treatment [6], as well as traces of HBV-DNA remaining detectable years after clinical recovery from acute hepatitis [7, 8], indicate that HBV cccDNA has an extremely long half-life in the human liver. Current therapies against HBV infection are effective at suppressing viral replication and improve long-term outcome [9, 10], but do not affect HBV cccDNA transcription template [11].

Although many steps of the HBV life cycle have been now well studied, the mechanisms of HBV cccDNA formation and regulation, as well as its regulatory interplay with the host immune system, are still poorly understood. While cell culture models are valuable to characterize defined aspects of the viral life cycle, *in vivo* models are invaluable to study the fate of HBV cccDNA during long-term chronic infection and to evaluate new antiviral strategies, including immune-therapies. HBV has an extremely narrow host range since it only infects hominoid apes, including chimpanzees. The latter have been used in pivotal studies deciphering host responses during acute HBV infection [12, 13] but are no longer

available for experimental studies [14]. The use of Macaques, relevant for toxicology studies, is under investigation as an alternative *in vivo* model of HBV infection but economic reasons retrain their use [15].

Mice, considered as a less expensive alternative animal model, are naturally not susceptible to HBV infection since the mouse sodium taurocholate cotransporting polypeptide (NTCP) orthologue does not allow HBV entry into murine hepatocytes [16, 17]. Mice or murine cell lines over-expressing the human NTCP can efficiently be infected by Hepatitis Delta virus (HDV) particles that share the same envelop as HBV and therefore enter by similar pathway. However, HBV replication was not detected and HBV cccDNA formation was suggested to be restricted in mouse cells [17-20]. To circumvent this issue, different alternatives have been proposed. Immune-deficient mice have been used to generate humanized liver models (HuHep mice) that are susceptible to HBV infection [21]. However, the absence of a functional immune system in these animals prevents the study of immunological issues regarding HBV infection, as well as the evaluation of novel immune-therapies. The injection of HBV minicircle or viral vectors in immune-competent mice was proposed to bypass limiting steps (i.e., entry and the HBV cccDNA formation) and allow transcription of HBV RNAs and virus production. Indeed, transfection of HBV minicircle led to the formation of HBV cccDNA-like molecules in hepatocytes and to persistent HBV replication in vivo [22, 23]. Chronic HBV infection has also been successfully established in immune-competent mice by inoculating low doses of adenovirus- [24] or adeno-associated virus (AAV) vectors containing the HBV genome [25-27]. These models have proven useful for immunological studies. However, despite the fact that HBV cccDNA has been previously detected in HNF1a null HBV transgenic mice [28] and in a murine hepatic cell line derived from a hTGF-alpha transgenic mouse that harbor an inducible HBV genome integration [29], it was assumed that its formation and maintenance would not occur in AAV-HBV-transduced mice, as it was reported for humanized NTCP transgenic mice [17].

Here, we investigated the establishment of an HBV cccDNA pool in HBV AAV8-HBVtransduced C57BL6 mice in comparison with HBV-infected HuHep mice and HBV-infected HepG2-NTCP cells. Intrahepatic DNA was extracted following a Hirt procedure that favors the enrichment of low molecular weight DNA such as the HBV cccDNA (see supplementary material and methods). It was then subjected to Southern blot analysis, the gold standard method to specifically detect HBV cccDNA [30]. Different HBV DNA forms were theoretically expected to be detected in AAV-HBV transduced cells. Those include the HBV polymerasefree rcDNA, the single-stranded (ss) AAV-HBV DNA (from incoming AAV particles), the episomal circular double-stranded (ds) AAV-HBV DNA monomers and multimers (formed by recombination, [31, 32]) as well as, hypothetically, the HBV cccDNA formed by re-import of HBV rcDNA from neo-formed cytoplasmic nucleocapsids (Figure 1A). The HBV DNA circular forms should theoretically all be linearized upon digestion at the unique XhoI site (Figure 1A). HBV rcDNA and cccDNA were detected at their respective expected size (given their agarose mobility properties according to their relaxed or supercoiled state) in HBV-infected-HuHep mice and -HepG2-NTCP cells (Figure 1B, lanes 1 and 5). As expected (Figure 1A), XhoI digestion resulted in a single 3,2 kb band corresponding to a double stranded linear (DSL) HBV DNA (Figure 1B, lanes 2 and 4). Interestingly, we also observed signals corresponding to the HBV rcDNA and cccDNA forms in intrahepatic DNA extracted from an AAV-HBVtransduced C57BL6 mouse (Figure 1B, lane 6). Importantly, these forms were not detected with a specific DIG-labeled AAV-vector probes (Figure 1B, lane 12). Additional bands with mobility properties around 1.4 kb, 1.7 kb, 3.4 kb, and 4.8 kb (Figure 1B, lanes 6, #) were observed in the AAV-HBV-transduced C57BL6 mouse sample but not in the HBV-infected-HepG2-NTCP or -HuHep mouse samples. These additional bands corresponded to AAV sequences as confirmed by hybridization with specific AAV DIG-labeled probes (**Figure 1B**, **lane 12**). The 4.8 kb and 1.4 kb bands corresponded to the AAV-HBV dsIDNA and ssDNA, respectively (**Figure 1A**). The two intermediate bands migrating at a 3.4 and 1.7 kb position, probably corresponded to circular episomal AAV-HBV monomers containing either a fulllength or a truncated AAV-HBV genome, respectively. Detection of all these HBV DNA forms, including cccDNA, was confirmed in different AAV-HBV-transduced C57BL6 mice (coming from different laboratories but transduced with the same AAV8-HBV vector [25]) (**Figure 1C**). Digestion with an HBV single cutter mainly resulted in a 3.2 kb band corresponding to the HBV dsIDNA and in a 4.8 kb band corresponding to the AAV-HBV dsIDNA, while leaving AAV-HBV ssDNA intact (**Figure 1C, Xhol**). The higher MW forms visible above the AAV-HBV dsIDNA after digestion with Xhol were most likely AAV-HBV concatemers. While Southern blot analyses allow to distinguish between the different HBV DNA forms, the

sensitivity of the technique is rather low (around 7,5 x 10⁴ copies with our method), time consuming and less reproducible than selective qPCR methods. In addition, if true discrimination of the HBV cccDNA from the almost identical viral linear DNA or rcDNA is still challenging, discrimination of HBV cccDNA from the AAV-HBV episome is even more challenging, as they not only share common sequences but also are both episomal forms. To increase the specificity of HBV cccDNA detection, a nuclease digestion is usually performed before selective qPCR methods based on the use of primers (and probes) spanning the nick in the HBV rcDNA and hybridizing to its "gap region". T5 exonuclease, that degrades HBV rcDNA but should leave episomal HBV cccDNA molecules intact, is currently widely used [33]. Accordingly, the cccDNA band was still detected in AAV-HBV samples after digestion

with T5 (Fig.2B, Iane 6). In addition, as expected, the circular closed AAV-HBV episome was also resistant to the T5 exonuclease activity (Figure 2A, and 2B lanes 6 and 13). Surprisingly, the AAV-HBV ssDNA band was also unaffected by T5 digestion (Figure 2B, Ianes 6 and 13). This latter might be due to the complex secondary structure formed by the AAV ITRs that have been shown to be less accessible for DNA polymerases for instance [34]. To specifically detect only HBV cccDNA, we used a combination of digestion with Xmal and SacI restriction enzymes, that cut only in the AAV-HBV vector (Figure S1, 2A, and 2B lanes 5 and 12), followed by incubation with the T5 exonuclease resulting in complete degradation of all the AAV DNA species, including HBV-AAV SS forms (likely due to the cut in the ITRs by Xmal) (Figure 2A, 2B lanes 7 and 14). We confirmed that this triple digestion allowed to detect cccDNA by qPCR by comparing three different DNA samples (one extracted from HBV-infected HepG2-NTCP cells and two extracted from AAV-HBV-transduced C57BL6 mouse livers) containing different amounts of total HBV DNA and cccDNA (Figure 2C).

Overall, we demonstrated that an HBV 3.2 kb cccDNA can be genuinely detected in livers from AAV-HBV-transduced C57BL6 mice that display a chronic HBV infection (as shown by stable secretions of HBeAg, HBsAg and viremia (**Figure S2**)). Until now, HBV cccDNA detection in mouse cells was reported in only two studies using HNF1a null HBV transgenic mice [28] or a murine hepatic cell line derived from a hTGF-alpha transgenic mouse that harbors an inducible and integrated HBV genome [29]. The advantage of the AAV-HBVtransduced mouse model over those latter models is that (i) different HBV genotypes or mutants can be easily inserted into the AAV vectors and (ii) different mouse strains with different genetic background can be used. The model will further allow studies to determine the half-life of HBV cccDNA and to understand if the presence of HBV cccDNA may influence

and explain the outcome of the infection after AAV-HBV transduction in different mouse strains [35]. As the mouse NTCP orthologue does not allow HBV entry into cells [17], HBV propagation cannot occur in AAV-HBV transduced C57BL6 mice. This suggests that HBV cccDNA might be formed by recycling of de novo-formed cytoplasmic nucleocapsids in AAV-HBV transduced mouse hepatocytes. If so, the model would allow to (i) identify the factors that may influence HBV cccDNA formation by recycling of newly-formed cytoplasmic nucleocapsids (ii) to study the impact of nucleos(t)ides analogues or core protein allosteric modulators [36] on HBV cccDNA recycling within chronically infected hepatocytes and determine whether a combination of these two classes of antiviral agents could help depleting the pool of intrahepatic HBV cccDNA [11]. Alternatively, HBV cccDNA could also be formed by recombination from the AAV-HBV episome. This could explain, at least in part, why HBV cccDNA was not detected in HBV-transgenic C57BL6 mice [37]. But this remains to be adequately investigated by long-term studies using nucleoside analogues to prevent potential recycling and/or AAV-HBV vector with mutation on ATGs of HBc gene to altogether prevent nucleocapsid formation. If cccDNA originates from genuine recycling the model will be useful to study both recycling itself and molecules, which could interfere with it. If cccDNA originates from recombination, then the model will yet be useful to study molecules capable to silence and/or induce degradation of cccDNA. It also remains to be determined if the detected cccDNA is functional and can genuinely lead to viral RNA synthesis, as well as if it would eventually take over the AAV-HBV episome as the main template for viral transcription. These hypotheses are so far indirectly supported by a recent study describing the disappearance of AAV-HBV and AAV-HDV episomes in transduced C57BL6 mice without a significant decline of HBV viral load [38].

In conclusion, our results highlight the relevance of the AAV-HBV/mouse model that will be pivotal to decipher the mechanisms of HBV cccDNA maintenance and regulation as well as to evaluate the fate of HBV cccDNA under novel therapies combining direct acting antivirals with immune-modulatory drugs for instance [39, 40].

Acknowledgments and conflict of interest

The academic laboratories are supported by grants from ANRS (French national agency for research on AIDS and viral hepatitis; grants from CSS4/CSS7 study committee), INSERM, the DEVweCAN LABEX (ANR-10-LABX-0061) and 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), the EU Infect ERA HBVccc and the LabEx HEPSYS. The present work has been supported by the "Sanofi-INSERM HBV Cure program" of Inserm, CIRI, CRCL and Sanofi (confer to the following link; http://www.anrs.fr/Hepatites-virales-B-et-C/Recherche-

fondamentale/Actualites/Communique-de-presse-SANOFI-INSERM-HBV-Cure-program-unealliance-medicale-academique-et-pharmaceutique-pour-la-recherche-sur-l-hepatite-B). The authors thanks Judith Fresquet, Audrey Diederichs, Laura Dimier, Nicolas Brignon, Halim Guerraoui and Virginie Archimbaud and for their technical assistance. We thank the Pasteur Institute (France) for providing pAAV-HBV1.2 and the Plateforme de Thérapie Génique in Nantes (France) for the production of the *in vivo* certified lots of AAV-HBV vectors and the adeno-uPA vector. We thank Jean-François Henry, Nadine Aguilera and Jean-Louis Thoumas from the animal facility (PBES, Plateau de Biologie Experimental de la Souris, ENS de Lyon) as well as Anaïs Ollivier for their technical help in handling mice.

References

- 1. Newbold, J.E., et al., The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J Virol, 1995. **69**(6): p. 3350-7.
- 2. Bock, C.T., et al., *Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. Virus Genes, 1994.* **8**(3): p. 215-29.
 - 3. Bock, C.T., et al., Structural organization of the hepatitis B virus minichromosome. J Mol Biol, 2001. **307**(1): p. 183-96.
- 4. Pollicino, T., 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**(3): p. 823-37.
- 5. Seeger, C., Zoulim, F., Mason, W., *Molecular Biology of hepatitis B viruses*, in *Fields Virology*, D.K.a.P. Howley, Editor. 2013, Lippincott Williams and Wilkins.
- 6. Zoulim, F. and S. Locarnini, *Hepatitis B virus resistance to nucleos(t)ide analogues*. Gastroenterology, 2009. **137**(5): p. 1593-608 e1-2.
- 7. Rehermann, B., et al., The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med, 1996. **2**(10): p. 1104-8.
- 8. Maynard, M., et al., Sustained HBs seroconversion during lamivudine and adefovir dipivoxil combination therapy for lamivudine failure. J Hepatol, 2005. **42**(2): p. 279-81.
- 9. Marcellin, P., et al., Adefovir dipivoxil for the treatment of hepatitis B e antigenpositive chronic hepatitis B. N Engl J Med, 2003. **348**(9): p. 808-16.
- 10. Zoulim, F., F. Lebosse, and M. Levrero, *Current treatments for chronic hepatitis B virus infections*. Curr Opin Virol, 2016. **18**: p. 109-16.
- 11. Boyd, A., et al., Decay of ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-HBV co-infected patients. J Hepatol, 2016. **65**(4): p. 683-91.
- 12. Guidotti, L.G., et al., Viral clearance without destruction of infected cells during acute HBV infection. Science, 1999. **284**(5415): p. 825-9.
- 13. Wieland, S., et al., *Genomic analysis of the host response to hepatitis B virus infection*. Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6669-74.
- 14. Harrington, M., State of the (research) chimp. Lab Anim (NY), 2012. **41**(2): p. 31.
- 15. Dupinay, T., et al., Discovery of naturally occurring transmissible chronic hepatitis B virus infection among Macaca fascicularis from Mauritius Island. Hepatology, 2013. **58**(5): p. 1610-20.
- 16. Yan, H., et al., Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife, 2012. **1**: p. e00049.
- 17. Lempp, F.A., et al., Evidence that hepatitis B virus replication in mouse cells is limited by the lack of a host cell dependency factor. J Hepatol, 2016. **64**(3): p. 556-64.
- 18. He, W., et al., Hepatitis D Virus Infection of Mice Expressing Human Sodium Taurocholate Co-transporting Polypeptide. PLoS Pathog, 2015. **11**(4): p. e1004840.
- 19. Lempp, F.A., et al., Sodium taurocholate cotransporting polypeptide is the limiting host factor of Hepatitis B Virus infection in macaque and pig hepatocytes. Hepatology, 2017.

Allweiss, L. and M. Dandri, Experimental in vitro and in vivo models for the study of 21. 606 human hepatitis B virus infection. J Hepatol, 2016. 64(1 Suppl): p. S17-31. 607 Guo, X., et al., The recombined cccDNA produced using minicircle technology 22. 608 609 mimicked HBV genome in structure and function closely. Sci Rep, 2016. 6: p. 25552. 610 23. Yan, Z., et al., HBVcircle: A novel tool to investigate hepatitis B virus covalently closed 611 circular DNA. J Hepatol, 2017. 66(6): p. 1149-1157. 612 24. Huang, L.R., et al., Transfer of HBV genomes using low doses of adenovirus vectors 613 leads to persistent infection in immune competent mice. Gastroenterology, 2012. 614 **142**(7): p. 1447-50 e3. 615 25. Dion, S., et al., Adeno-associated virus-mediated gene transfer leads to persistent 616 hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. J 617 618 Virol, 2013. 87(10): p. 5554-63. 619 26. Yang, D., et al., A mouse model for HBV immunotolerance and immunotherapy. Cell 620 Mol Immunol, 2014. **11**(1): p. 71-8. 621 27. Ye, L., et al., Adeno-Associated Virus Vector Mediated Delivery of the HBV Genome 622 Induces Chronic Hepatitis B Virus Infection and Liver Fibrosis in Mice. PLoS One, 2015. 623 **10**(6): p. e0130052. 624 28. Raney, A.K., et al., Nuclear covalently closed circular viral genomic DNA in the liver of 625 hepatocyte nuclear factor 1 alpha-null hepatitis B virus transgenic mice. J Virol, 2001. 626 627 **75**(6): p. 2900-11. 628 29. Cui, X., J.T. Guo, and J. Hu, Hepatitis B Virus Covalently Closed Circular DNA Formation 629 in Immortalized Mouse Hepatocytes Associated with Nucleocapsid Destabilization. J 630 Virol, 2015. 89(17): p. 9021-8. 631 30. Nassal, M., HBV cccDNA: viral persistence reservoir and key obstacle for a cure of 632 chronic hepatitis B. Gut, 2015. 64(12): p. 1972-84. 633 31. Nakai, H., T.A. Storm, and M.A. Kay, Recruitment of single-stranded recombinant 634 adeno-associated virus vector genomes and intermolecular recombination are 635 636 responsible for stable transduction of liver in vivo. J Virol, 2000. 74(20): p. 9451-63. 637 32. Nakai, H., et al., Extrachromosomal recombinant adeno-associated virus vector 638 genomes are primarily responsible for stable liver transduction in vivo. J Virol, 2001. 639 **75**(15): p. 6969-76. 640 33. Niu, C., et al., The Smc5/6 Complex Restricts HBV when Localized to ND10 without 641 Inducing an Innate Immune Response and Is Counteracted by the HBV X Protein 642 Shortly after Infection. PLoS One, 2017. **12**(1): p. e0169648. 643 34. 644 Fagone, P., et al., Systemic errors in quantitative polymerase chain reaction titration 645 of self-complementary adeno-associated viral vectors and improved alternative 646 methods. Hum Gene Ther Methods, 2012. 23(1): p. 1-7. 647 35. Chou, H.H., et al., Age-related immune clearance of hepatitis B virus infection requires 648 the establishment of gut microbiota. Proc Natl Acad Sci U S A, 2015. 112(7): p. 2175-649 80. 650 36. Durantel, D. and F. Zoulim, New antiviral targets for innovative treatment concepts 651 for hepatitis B virus and hepatitis delta virus. J Hepatol, 2016. 64(1 Suppl): p. S117-31. 652 653 37. Guidotti, L.G., et al., High-level hepatitis B virus replication in transgenic mice. J Virol, 654 1995. **69**(10): p. 6158-69. 655 656 657 658 11 659 660

Mailly, L., M.B. Zeisel, and T.F. Baumert, Towards novel immunocompetent animal

models for hepatitis B virus infection. Hepatology, 2017.

601 602 603

604

605

20.

662			
663	38	Suarez-Amaran I et al A new HDV mouse model identifies mitochondrial antivir	al
664	00.	signaling protein (MAVS) as a key player in JEN bata induction. Hepatol. 2017	л
665	00	signaling protein (MAVS) as a key player in involution. J Hepatol, 2017.	
666	39.	Bian, Y., et al., Vaccines Targeting Pres1 Domain Overcome Immune Tolerance in HB	V
667		Carrier Mice. Hepatology, 2017.	
668	40.	Martin, P., et al., TG1050, an immunotherapeutic to treat chronic hepatitis B, induce	2S
669		robust T cells and exerts an antiviral effect in HBV-persistent mice. Gut, 2015. 64 (12):
670		n 1961-71	·
671		p. 1701 7 1.	
672			
673			
674			
675			
676			
677			
679			
670			
690			
000			
081			
682			
683			
684			
685			
686			
687			
688			
689			
690			
691			
692			
693			
694			
695			
696			
697			
698			
699			
700			
701			
702			
703			
700			
705			
705			
700			
707			
700			
709			
710			
711			
/12			
/13			
/14			
/15			
/16			
717			
718			
719		1	.2
720			

Figure legends

Figure 1: **Detection of the HBV cccDNA in AAV-HBV transduced C57BL6 mice**. (A) Schematic representation of HBV DNA forms extracted by a Hirt procedure and their expected modifications after Xhol digestion. (B) Intrahepatic DNAs from an HBV-infected HuHep mouse (day 84 p.i.), an AAV-HBV-transduced C57BL6/J mouse (day 28 p.t.) or HBV-infected HepG2-NTCP cells (day 10 p.i.) were extracted following a Hirt procedure and submitted to Southern blot analyses using HBV-DIG or AAV-DIG labeled probes. (C) Intrahepatic DNAs from 20 AAV-HBV-transduced C57BL6/N mice (day 84 p.t.) were extracted following an Hirt procedure, pooled in four different groups (#1, #2, #3, #4), digested or not by Xhol and subjected to Southern blot analyses using HBV-DIG or AAV-DIG or AAV-DIG labeled probes. ND: not digested, MW: molecular weight.

Figure 2: Strategy to distinguish between the HBV cccDNA and the AAV-HBV episome in AAV-HBV transduced C57BL6 mice. (A) Schematic representation of HBV DNA forms extracted by a Hirt procedure and their expected behavior after the indicated digestions. (B) Intrahepatique DNAs from an AAV-HBV-transduced C57BL6/N mouse (day 28 p.t.) or HBVinfected HepG2-NTCP cells (day 10 p.i) were extracted following a Hirt procedure, digested or not by indicated restriction enzymes and subjected to Southern blot analyses using HBV-DIG or AAV-DIG labeled probes. (C) Intrahepatic DNAs from two AAV-HBV-transduced C57BL6/J mice sacrificed respectively at day 21 p.t and day 28 p.t or HBV-infected HepG2-NTCP cells (day 10 p.i) were extracted following a Hirt procedure, digested or not by Xmal, SacI and T5 and subjected to Southern blot analyses using HBV-DIG, AAV-DIG labeled probes (left panels) or specific qPCR analyses to quantify total HBV DNA or cccDNA (right panels).

ND: not digested, MW: molecular weight. *not digested with no digestion buffer or incubation at 37°C.

	no digestion (ND)	Xhol digestion
HBV rcDNA	\bigcirc	dsl HBV (3,2 kb)
HBV cccDNA	supercoiled	dsl HBV (3,2 kb)
AAV-HBV episome		dsl AAV-HBV (4,8 kb)
ss AAV-HBV	F	├ ────╡

В

Α



AAV-DIG labeled probes



Α

Detection and quantification of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector

Julie Lucifora¹, Anna Salvetti¹, Xavier Marniquet², Laurent Mailly³, Barbara Testoni¹, Floriane Fusil⁴, Aurore Inchauspé^{1,2}, Maud Michelet¹, Marie-Louise Michel⁵, Massimo Levrero¹, Pierre Cortez², Thomas F. Baumert^{3,6}, François-Loic Cosset⁴, Cécile Challier², Fabien Zoulim^{1,7,8,#} and David Durantel^{1,#}

¹INSERM, U1052, Cancer Research Center of Lyon (CRCL), Université de Lyon (UCBL1), CNRS UMR_5286, Centre Léon Bérard, Lyon, France ;
²Sanofi R&D, Infectious Disease Therapeutic Area, Marcy l'Etoile, France ;
³INSERM U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, Strasbourg, France ;
⁴INSERM, U1111, International Center for Infectiology Research (CIRI), Team EVIR, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, Ecole Normale Supérieure de Lyon, Univ Lyon, F-69007, Lyon, France
⁵INSERM U994, Institut Pasteur, Paris, France
⁶Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Hôpitaux Universitaires de Strasbourg, Strasbourg, France
⁸Institut Universitaire de France (IUF), Paris, France

Supplementary material and method

Virus production and cell culture. HBV inoculum was prepared from HepAD38 [41] supernatants by polyethylene-glycol-MW-8000 (PEG8000, SIGMA) precipitation (8% final). Viral stock with a titer reaching at least 1x10¹⁰ vge/mL was tested endotoxin free. Recombinant AAV8-HBV vectors carrying 1.2 copies of the HBV genome (genotype D) were produced (using the pAAV-HBV1.2 plasmid provided by the Pasteur Institute (France)) and titrated by the "Plateforme de Thérapie Génique" in Nantes, France (INSERM U1089) and titrated by qPCR, as previously described [25]. HepG2-NTCP cells were seeded at 10⁵ cells/cm² in DMEM medium supplemented with penicillin (Life Technology), streptomycin (Life Technology), sodium pyruvate (Life Technology), 5% Fetal Calf Serum (FCS; Fetalclone

II[™], PERBIO). The day after, medium was renewed and complemented with 2,5% DMSO (SIGMA) and cells were infected at a multiplicity of infection of 250 in the presence of 4% PEG800 three days later for at least 16h. Cells were then washed with PBS and maintained in medium containing DMSO until lysis for analyses.

Mouse experiments.

Sanofi's site. All *in vivo* experiments were made accordingly to French and European regulations on animal welfare and Public Health Service recommendations and all protocols have been reviewed and approved by the institutional animal care committee of Sanofi (APAFIS#2189-2015100714329651v1). All animals were housed in a specific-pathogen-free environment in the animal facilities of Sanofi, Marcy l'Etoile, France. Eight-week-old C57BL6/J female mice (Charles River, Les Oncins, Saint-Germain Nuelles, France) received a single tail vein injection of 5.10¹⁰ vg/mouse of AAV8-HBV viral particles. After 21 or 28 days post-injection, mice were euthanized, blood was collected and liver pieces were flash frozen in liquid nitrogen and kept at -80°C before further processing. HBe antigens level in the serum of mice #23 (day 21 p.t.) and #32 (day 28 p.t.) used in **figure 1B and 2C** reached 60 NCU/mL and 2100 NCU/mL respectively (Autobio kit according to the manufacturer's instructions (AutoBio, China)).

<u>University of Strasbourg's site.</u> All animal were housed in the A3 animal facility of the Inserm U1110, Research Institute of Viral and Liver Disease. The procedures were approved by the local ethic committee and authorized by the French ministry of research (n° 02014120511054408 – APAFIS#74.03). Twenty 8-week old C57BL6/N male mice (Charles River, Les Oncins, Saint-Germain Nuelles, France) were injected intravenously with 10¹¹ vge of AAV8-HBV and sacrificed 12 weeks later. Liver pieces were flash frozen in liquid nitrogen

and kept at -80°C before further processing. Sera analysis were pooled in four groups with HBeAg levels reaching 1024 +/- 219, 987 +/- 251, 725 +/- 254 and 739 +/-307 NCU/mL for group #1, #2, #3 and #4 respectively (using the Autobio kit according to the manufacturer's instructions (AutoBio, China)).

<u>CIRI's site</u>. All experiments 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). Primary Human Hepatocytes (PHH, Corning, BD Gentest) were injected in FRG mice intrasplenically 48h after adeno-uPA conditioning [42]. Mice were subjected to NTBC cycling during the liver repopulation process. 20 weeks-old humanized FRG (or 9 weeks post engraftment of PHH) female mice with HSA levels >19 mg/ml, as determined using a Cobas C501 analyzer, Roche Applied Science, were infected by IP injection with 5x10⁸ vge/mL of HBV. Mice were sacrificed 11 weeks post-infection. Liver pieces were flash frozen in liquid nitrogen and kept at -80°C before further processing. HBe antigens level in the serum of mouse #473 used in **figure 1B** reached 3300 NCU/mL (Autobio kit according to the manufacturer's instructions (AutoBio, China)).

Hirt procedure and Southern Blot analyses.

DNA were extracted following a modified Hirt procedure [43]. 80 ug (for mice samples) or 20 ug (for cellular samples) of DNA were subjected to Southern blot analyses using mixes of DIG-labeled probes (synthesized using primers listed below and the "PCR DIG probe synthesis kit" (Roche)), an AP conjugated anti-DIG antibody (Roche) and CDP-Star[®] (Roche) according to the manufacturer's instructions.

Target	Name	Sequence
HBV	HBV-F1	TAGCGCCTCATTTTGTGGGT
HBV	HBV-R1	CTTCCTGTCTGGCGATTGGT
HBV	HBV-F2	TAGGACCCCTTCTCGTGTTA
HBV	HBV-R2	CCGTCCGAAGGTTTGGTACA
HBV	HBV-F3	ATGTGGTATTGGGGGGCCAAG
HBV	HBV-R3	GGTTGCGTCAGCAAACACTT
HBV	HBV-F4	TGGACCTTTTCGGCTCCTC
HBV	HBV-R4	GGGAGTCCGCGTAAAGAGAG
HBV	HBV-F5	GTCTGTGCCTTCTCATCTG
HBV	HBV-R5	AGGAGACTCTAAGGCTTCC
HBV	HBV-F6	TACTGCACTCAGGCAAGCAA
HBV	HBV-R6	TGCGAATCCACACTCCGAAA
HBV	HBV-F8	AGACGAAGGTCTCAATCGCC
HBV	HBV-R8	ACCCACAAAATGAGGCGCTA
AAV	AAV.D-F1	CTCCATCACTAGGGGTTCCT
AAV	AAV-R1	CAATTCGCCCTATAGTGAGT
AAV	AAV-R2	GTTCGAAATCGATAAGCTTGG

Quantitative PCR analyses. One microgram of Hirt extracted DNA were digested or not for 2h at 37°C with 2,5 U of Xmal (New England Biolabs) and 5 U of SacI-HF[®] (New England Biolabs). Ten unit of T5 exonuclease (New England Biolabs) were added and DNA were further incubated at 37°C for 30 min and 30 min at 70°C. DNA were then diluted in water to

reach a concentration of 10 ng/uL. Forty nanogram of DNA were used to measure the HBV

DNA and cccDNA amounts using specific TaqMan probes and primers as described before

[44].

Supplementary References

- 41. Ladner, S.K., et al., Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother, 1997. **41**(8): p. 1715-20.
- 42. Bissig, K.D., et al., *Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model*. Proc Natl Acad Sci U S A, 2007. **104**(51): p. 20507-11.
- 43. Gao, W. and J. Hu, Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. J Virol, 2007. **81**(12): p. 6164-74.
- 44. Allweiss, L., et al., Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. Gut, 2017.

Supplementary Figure



Figure S1

XhoI (2189)





Twelve C57BL6/J mice received a single tail vein injection of 5.10¹⁰ vg/mouse of AAV8-HBV viral particles. At the indicated time post-transduction, blood was collected and levels HBeAg, HBsAg and viremia were determined by ELISA and qPCR respectively.