



HAL
open science

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 Maily, 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 Maily, 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.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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-Loïc 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 Hépatito-digestif, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

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

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

#Corresponding authors

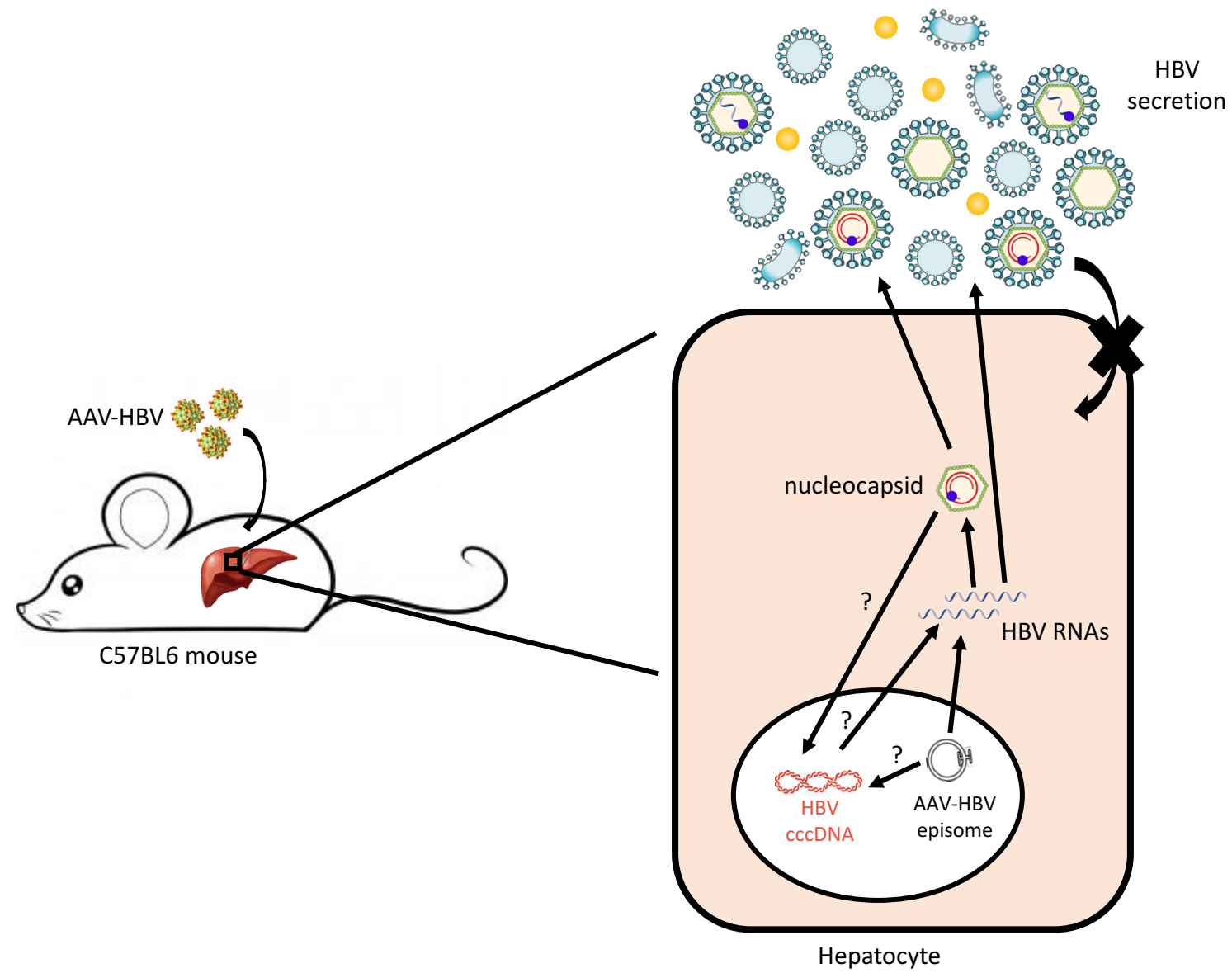
Dr. David Durantel and Prof. Fabien Zoulim

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 : david.durantel@inserm.fr ; fabien.zoulim@inserm.fr

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*

61
62
63 **Abstract**
64

65 Hepatitis B Virus (HBV) persists in infected hepatocytes as an episomal covalently-closed-
66 circular DNA mini-chromosome, called cccDNA. As the main nuclear transcription template,
67
68 HBV cccDNA is a key replication intermediate in the viral life cycle. Little is known about the
69
70 mechanisms involved in its formation, maintenance and fate under antiviral therapies. This is
71
72 mainly due to the lack of small immune-competent animal models able to recapitulate the
73
74 entire HBV replication cycle, including formation of HBV cccDNA. Here we report that HBV
75
76 cccDNA can be detected by Southern blot analyses in the liver of C57BL6 mice transduced
77
78 with AAV-HBV. HBV cccDNA persists in the liver of these animals together with the AAV-HBV
79
80 episome. We also set up a PCR strategy to distinguish the HBV cccDNA from the AAV-HBV
81
82 episome. These suggest that the AAV-HBV/mouse model might be relevant to test drugs
83
84 targeting HBV cccDNA regulation and persistence.
85
86
87
88
89
90
91

92 **Keywords**
93

94 Hepatitis B virus, cccDNA, adeno-associated virus, immune-competent mouse, immune-
95
96 therapeutics
97
98
99

100 **Highlights**
101

- 102 • The HBV cccDNA was detected by Southern blotting in AAV-HBV transduced C57BL6
103 mice
104
- 105 • A strategy was set up to distinguish the HBV cccDNA from the AAV-HBV episome by
106 qPCR
107
- 108 • AAV-HBV transduced immune-competent mice will allow to test drugs targeting
109 cccDNA regulation and stability *in vivo*
110
111
112
113
114
115
116
117
118
119
120

121
122
123 **Main text**
124

125 The Hepatitis B virus (HBV) circulates in the blood of patients as virions containing a partially
126 double stranded relaxed circular DNA (rcDNA). However, the virus persists in the nucleus of
127 infected hepatocytes as a covalently-closed-circular DNA, called HBV cccDNA, which is the
128 template for all viral RNAs production. HBV cccDNA is organized in a chromatin-like structure
129 that displays a typical beads-on-a-string arrangement by electron microscopy [1, 2]. Host
130 histone proteins as well as other proteins involved in gene expressing regulation are bound
131 to HBV cccDNA [3] and its transcriptional activity is subjected to the “histone code” [4]. Since
132 it does not contain an origin of replication, HBV cccDNA persistence relies on its stability in
133 non-dividing cells and/or on its replenishment via re-import of rcDNA from neo-synthesized
134 nucleocapsids [5]. The universal rebound of HBV replication upon withdrawal from
135 nucleos(t)ide analogue treatment [6], as well as traces of HBV-DNA remaining detectable
136 years after clinical recovery from acute hepatitis [7, 8], indicate that HBV cccDNA has an
137 extremely long half-life in the human liver. Current therapies against HBV infection are
138 effective at suppressing viral replication and improve long-term outcome [9, 10], but do not
139 affect HBV cccDNA transcription template [11].
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158

159 Although many steps of the HBV life cycle have been now well studied, the mechanisms of
160 HBV cccDNA formation and regulation, as well as its regulatory interplay with the host
161 immune system, are still poorly understood. While cell culture models are valuable to
162 characterize defined aspects of the viral life cycle, *in vivo* models are invaluable to study the
163 fate of HBV cccDNA during long-term chronic infection and to evaluate new antiviral
164 strategies, including immune-therapies. HBV has an extremely narrow host range since it
165 only infects hominoid apes, including chimpanzees. The latter have been used in pivotal
166 studies deciphering host responses during acute HBV infection [12, 13] but are no longer
167
168
169
170
171
172
173
174
175
176
177
178
179
180

181
182
183 available for experimental studies [14]. The use of Macaques, relevant for toxicology studies,
184
185 is under investigation as an alternative *in vivo* model of HBV infection but economic reasons
186
187 retrain their use [15].
188

189
190 Mice, considered as a less expensive alternative animal model, are naturally not susceptible
191
192 to HBV infection since the mouse sodium taurocholate cotransporting polypeptide (NTCP)
193
194 orthologue does not allow HBV entry into murine hepatocytes [16, 17]. Mice or murine cell
195
196 lines over-expressing the human NTCP can efficiently be infected by Hepatitis Delta virus
197
198 (HDV) particles that share the same envelop as HBV and therefore enter by similar pathway.
199

200
201 However, HBV replication was not detected and HBV cccDNA formation was suggested to be
202
203 restricted in mouse cells [17-20]. To circumvent this issue, different alternatives have been
204
205 proposed. Immune-deficient mice have been used to generate humanized liver models
206
207 (HuHep mice) that are susceptible to HBV infection [21]. However, the absence of a
208
209 functional immune system in these animals prevents the study of immunological issues
210
211 regarding HBV infection, as well as the evaluation of novel immune-therapies. The injection
212
213 of HBV minicircle or viral vectors in immune-competent mice was proposed to bypass
214
215 limiting steps (i.e., entry and the HBV cccDNA formation) and allow transcription of HBV
216
217 RNAs and virus production. Indeed, transfection of HBV minicircle led to the formation of
218
219 HBV cccDNA-like molecules in hepatocytes and to persistent HBV replication *in vivo* [22, 23].
220
221

222
223 Chronic HBV infection has also been successfully established in immune-competent mice by
224
225 inoculating low doses of adenovirus- [24] or adeno-associated virus (AAV) vectors containing
226
227 the HBV genome [25-27]. These models have proven useful for immunological studies.
228
229

230
231 However, despite the fact that HBV cccDNA has been previously detected in HNF1a null HBV
232
233 transgenic mice [28] and in a murine hepatic cell line derived from a hTGF-alpha transgenic
234
235 mouse that harbor an inducible HBV genome integration [29], it was assumed that its
236
237

241
242
243 formation and maintenance would not occur in AAV-HBV-transduced mice, as it was
244
245 reported for humanized NTCP transgenic mice [17].
246
247
248

249
250 Here, we investigated the establishment of an HBV cccDNA pool in HBV AAV8-HBV-
251
252 transduced C57BL6 mice in comparison with HBV-infected HuHep mice and HBV-infected
253
254 HepG2-NTCP cells. Intrahepatic DNA was extracted following a Hirt procedure that favors
255
256 the enrichment of low molecular weight DNA such as the HBV cccDNA (see supplementary
257
258 material and methods). It was then subjected to Southern blot analysis, the gold standard
259
260 method to specifically detect HBV cccDNA [30]. Different HBV DNA forms were theoretically
261
262 expected to be detected in AAV-HBV transduced cells. Those include the HBV polymerase-
263
264 free rcDNA, the single-stranded (ss) AAV-HBV DNA (from incoming AAV particles), the
265
266 episomal circular double-stranded (ds) AAV-HBV DNA monomers and multimers (formed by
267
268 recombination, [31, 32]) as well as, hypothetically, the HBV cccDNA formed by re-import of
269
270 HBV rcDNA from neo-formed cytoplasmic nucleocapsids (**Figure 1A**). The HBV DNA circular
271
272 forms should theoretically all be linearized upon digestion at the unique XhoI site (**Figure**
273
274 **1A**). HBV rcDNA and cccDNA were detected at their respective expected size (given their
275
276 agarose mobility properties according to their relaxed or supercoiled state) in HBV-infected-
277
278 HuHep mice and -HepG2-NTCP cells (**Figure 1B, lanes 1 and 5**). As expected (**Figure 1A**), XhoI
279
280 digestion resulted in a single 3,2 kb band corresponding to a double stranded linear (DSL)
281
282 HBV DNA (**Figure 1B, lanes 2 and 4**). Interestingly, we also observed signals corresponding to
283
284 the HBV rcDNA and cccDNA forms in intrahepatic DNA extracted from an AAV-HBV-
285
286 transduced C57BL6 mouse (**Figure 1B, lane 6**). Importantly, these forms were not detected
287
288 with a specific DIG-labeled AAV-vector probes (**Figure 1B, lane 12**). Additional bands with
289
290 mobility properties around 1.4 kb, 1.7 kb, 3.4 kb, and 4.8 kb (**Figure 1B, lanes 6, #**) were
291
292
293
294
295
296
297
298
299
300

301
302
303 observed in the AAV-HBV-transduced C57BL6 mouse sample but not in the HBV-infected-
304
305 HepG2-NTCP or -HuHep mouse samples. These additional bands corresponded to AAV
306
307 sequences as confirmed by hybridization with specific AAV DIG-labeled probes (**Figure 1B,**
308
309 **lane 12**). The 4.8 kb and 1.4 kb bands corresponded to the AAV-HBV dsDNA and ssDNA,
310
311 respectively (**Figure 1A**). The two intermediate bands migrating at a 3.4 and 1.7 kb position,
312
313 probably corresponded to circular episomal AAV-HBV monomers containing either a full-
314
315 length or a truncated AAV-HBV genome, respectively. Detection of all these HBV DNA forms,
316
317 including cccDNA, was confirmed in different AAV-HBV-transduced C57BL6 mice (coming
318
319 from different laboratories but transduced with the same AAV8-HBV vector [25]) (**Figure 1C**).
320
321 Digestion with an HBV single cutter mainly resulted in a 3.2 kb band corresponding to the
322
323 HBV dsDNA and in a 4.8 kb band corresponding to the AAV-HBV dsDNA, while leaving AAV-
324
325 HBV ssDNA intact (**Figure 1C, XhoI**). The higher MW forms visible above the AAV-HBV
326
327 dsDNA after digestion with XhoI were most likely AAV-HBV concatemers.
328
329

330
331
332 While Southern blot analyses allow to distinguish between the different HBV DNA forms, the
333
334 sensitivity of the technique is rather low (around $7,5 \times 10^4$ copies with our method), time
335
336 consuming and less reproducible than selective qPCR methods. In addition, if true
337
338 discrimination of the HBV cccDNA from the almost identical viral linear DNA or rcDNA is still
339
340 challenging, discrimination of HBV cccDNA from the AAV-HBV episome is even more
341
342 challenging, as they not only share common sequences but also are both episomal forms. To
343
344 increase the specificity of HBV cccDNA detection, a nuclease digestion is usually performed
345
346 before selective qPCR methods based on the use of primers (and probes) spanning the nick
347
348 in the HBV rcDNA and hybridizing to its "gap region". T5 exonuclease, that degrades HBV
349
350 rcDNA but should leave episomal HBV cccDNA molecules intact, is currently widely used
351
352 [33]. Accordingly, the cccDNA band was still detected in AAV-HBV samples after digestion
353
354
355
356
357
358
359
360

361
362
363 with T5 (Fig.2B, lane 6). In addition, as expected, the circular closed AAV-HBV episome was
364
365 also resistant to the T5 exonuclease activity (**Figure 2A, and 2B lanes 6 and 13**). Surprisingly,
366
367 the AAV-HBV ssDNA band was also unaffected by T5 digestion (**Figure 2B, lanes 6 and 13**).
368
369 This latter might be due to the complex secondary structure formed by the AAV ITRs that
370
371 have been shown to be less accessible for DNA polymerases for instance [34]. To specifically
372
373 detect only HBV cccDNA, we used a combination of digestion with XmaI and SacI restriction
374
375 enzymes, that cut only in the AAV-HBV vector (**Figure S1, 2A, and 2B lanes 5 and 12**),
376
377 followed by incubation with the T5 exonuclease resulting in complete degradation of all the
378
379 AAV DNA species, including HBV-AAV SS forms (likely due to the cut in the ITRs by XmaI)
380
381 (**Figure 2A, 2B lanes 7 and 14**). We confirmed that this triple digestion allowed to detect
382
383 cccDNA by qPCR by comparing three different DNA samples (one extracted from HBV-
384
385 infected HepG2-NTCP cells and two extracted from AAV-HBV-transduced C57BL6 mouse
386
387 livers) containing different amounts of total HBV DNA and cccDNA (**Figure 2C**).

388
389
390
391
392
393
394
395 Overall, we demonstrated that an HBV 3.2 kb cccDNA can be genuinely detected in livers
396
397 from AAV-HBV-transduced C57BL6 mice that display a chronic HBV infection (as shown by
398
399 stable secretions of HBeAg, HBsAg and viremia (**Figure S2**)). Until now, HBV cccDNA
400
401 detection in mouse cells was reported in only two studies using HNF1a null HBV transgenic
402
403 mice [28] or a murine hepatic cell line derived from a hTGF-alpha transgenic mouse that
404
405 harbors an inducible and integrated HBV genome [29]. The advantage of the AAV-HBV-
406
407 transduced mouse model over those latter models is that (i) different HBV genotypes or
408
409 mutants can be easily inserted into the AAV vectors and (ii) different mouse strains with
410
411 different genetic background can be used. The model will further allow studies to determine
412
413 the half-life of HBV cccDNA and to understand if the presence of HBV cccDNA may influence
414
415
416
417
418
419
420

421
422
423 and explain the outcome of the infection after AAV-HBV transduction in different mouse
424
425 strains [35]. As the mouse NTCP orthologue does not allow HBV entry into cells [17], HBV
426
427 propagation cannot occur in AAV-HBV transduced C57BL6 mice. This suggests that HBV
428
429 cccDNA might be formed by recycling of *de novo*-formed cytoplasmic nucleocapsids in AAV-
430
431 HBV transduced mouse hepatocytes. If so, the model would allow to (i) identify the factors
432
433 that may influence HBV cccDNA formation by recycling of newly-formed cytoplasmic
434
435 nucleocapsids (ii) to study the impact of nucleos(t)ides analogues or core protein allosteric
436
437 modulators [36] on HBV cccDNA recycling within chronically infected hepatocytes and
438
439 determine whether a combination of these two classes of antiviral agents could help
440
441 depleting the pool of intrahepatic HBV cccDNA [11]. Alternatively, HBV cccDNA could also be
442
443 formed by recombination from the AAV-HBV episome. This could explain, at least in part,
444
445 why HBV cccDNA was not detected in HBV-transgenic C57BL6 mice [37]. But this remains to
446
447 be adequately investigated by long-term studies using nucleoside analogues to prevent
448
449 potential recycling and/or AAV-HBV vector with mutation on ATGs of HBc gene to altogether
450
451 prevent nucleocapsid formation. If cccDNA originates from genuine recycling the model will
452
453 be useful to study both recycling itself and molecules, which could interfere with it. If
454
455 cccDNA originates from recombination, then the model will yet be useful to study molecules
456
457 capable to silence and/or induce degradation of cccDNA. It also remains to be determined if
458
459 the detected cccDNA is functional and can genuinely lead to viral RNA synthesis, as well as if
460
461 it would eventually take over the AAV-HBV episome as the main template for viral
462
463 transcription. These hypotheses are so far indirectly supported by a recent study describing
464
465 the disappearance of AAV-HBV and AAV-HDV episomes in transduced C57BL6 mice without
466
467 a significant decline of HBV viral load [38].
468
469
470
471
472
473
474
475
476
477
478
479
480

481
482
483 In conclusion, our results highlight the relevance of the AAV-HBV/mouse model that will be
484
485 pivotal to decipher the mechanisms of HBV cccDNA maintenance and regulation as well as to
486
487 evaluate the fate of HBV cccDNA under novel therapies combining direct acting antivirals
488
489 with immune-modulatory drugs for instance [39, 40].
490
491

492 493 494 **Acknowledgments and conflict of interest**

495
496 The academic laboratories are supported by grants from ANRS (French national agency for
497
498 research on AIDS and viral hepatitis; grants from CSS4/CSS7 study committee), INSERM, the
499
500 DEVweCAN LABEX (ANR-10-LABX-0061) and the LabEx Ecofect (ANR-11-LABX-0048) of the
501
502 “Université de Lyon”, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007)
503
504 operated by the French National Research Agency (ANR), the EU Infect ERA HBVccc and the
505
506 LabEx HEPSYS. The present work has been supported by the “Sanofi-INSERM HBV Cure
507
508 program” of Inserm, CIRI, CRCL and Sanofi (confer to the following link;
509
510 [http://www.anrs.fr/Hepatites-virales-B-et-C/Recherche-](http://www.anrs.fr/Hepatites-virales-B-et-C/Recherche-fondamentale/Actualites/Communique-de-presse-SANOFI-INSERM-HBV-Cure-program-une-alliance-medicale-academique-et-pharmaceutique-pour-la-recherche-sur-l-hepatite-B)
511
512 [fondamentale/Actualites/Communique-de-presse-SANOFI-INSERM-HBV-Cure-program-une-](http://www.anrs.fr/Hepatites-virales-B-et-C/Recherche-fondamentale/Actualites/Communique-de-presse-SANOFI-INSERM-HBV-Cure-program-une-alliance-medicale-academique-et-pharmaceutique-pour-la-recherche-sur-l-hepatite-B)
513
514 [alliance-medicale-academique-et-pharmaceutique-pour-la-recherche-sur-l-hepatite-B](http://www.anrs.fr/Hepatites-virales-B-et-C/Recherche-fondamentale/Actualites/Communique-de-presse-SANOFI-INSERM-HBV-Cure-program-une-alliance-medicale-academique-et-pharmaceutique-pour-la-recherche-sur-l-hepatite-B)). The
515
516 authors thanks Judith Fresquet, Audrey Diederichs, Laura Dimier, Nicolas Brignon, Halim
517
518 Guerraoui and Virginie Archimbaud and for their technical assistance. We thank the Pasteur
519
520 Institute (France) for providing pAAV-HBV1.2 and the Plateforme de Thérapie Génique in
521
522 Nantes (France) for the production of the *in vivo* certified lots of AAV-HBV vectors and the
523
524 adeno-uPA vector. We thank Jean-François Henry, Nadine Aguilera and Jean-Louis Thoumas
525
526 from the animal facility (PBES, Plateau de Biologie Experimental de la Souris, ENS de Lyon) as
527
528 well as Anaïs Ollivier for their technical help in handling mice.
529
530
531
532
533
534
535
536
537
538
539
540

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. Rehmann, 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 antigen-positive 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.

- 601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
20. Maily, L., M.B. Zeisel, and T.F. Baumert, *Towards novel immunocompetent animal models for hepatitis B virus infection*. Hepatology, 2017.
 21. Allweiss, L. and M. Dandri, *Experimental in vitro and in vivo models for the study of human hepatitis B virus infection*. J Hepatol, 2016. **64**(1 Suppl): p. S17-31.
 22. Guo, X., et al., *The recombined cccDNA produced using minicircle technology mimicked HBV genome in structure and function closely*. Sci Rep, 2016. **6**: p. 25552.
 23. Yan, Z., et al., *HBVcircle: A novel tool to investigate hepatitis B virus covalently closed circular DNA*. J Hepatol, 2017. **66**(6): p. 1149-1157.
 24. Huang, L.R., et al., *Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice*. Gastroenterology, 2012. **142**(7): p. 1447-50 e3.
 25. Dion, S., et al., *Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules*. J Virol, 2013. **87**(10): p. 5554-63.
 26. Yang, D., et al., *A mouse model for HBV immunotolerance and immunotherapy*. Cell Mol Immunol, 2014. **11**(1): p. 71-8.
 27. Ye, L., et al., *Adeno-Associated Virus Vector Mediated Delivery of the HBV Genome Induces Chronic Hepatitis B Virus Infection and Liver Fibrosis in Mice*. PLoS One, 2015. **10**(6): p. e0130052.
 28. Raney, A.K., et al., *Nuclear covalently closed circular viral genomic DNA in the liver of hepatocyte nuclear factor 1 alpha-null hepatitis B virus transgenic mice*. J Virol, 2001. **75**(6): p. 2900-11.
 29. Cui, X., J.T. Guo, and J. Hu, *Hepatitis B Virus Covalently Closed Circular DNA Formation in Immortalized Mouse Hepatocytes Associated with Nucleocapsid Destabilization*. J Virol, 2015. **89**(17): p. 9021-8.
 30. Nassal, M., *HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B*. Gut, 2015. **64**(12): p. 1972-84.
 31. Nakai, H., T.A. Storm, and M.A. Kay, *Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver in vivo*. J Virol, 2000. **74**(20): p. 9451-63.
 32. Nakai, H., et al., *Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo*. J Virol, 2001. **75**(15): p. 6969-76.
 33. Niu, C., et al., *The Smc5/6 Complex Restricts HBV when Localized to ND10 without Inducing an Innate Immune Response and Is Counteracted by the HBV X Protein Shortly after Infection*. PLoS One, 2017. **12**(1): p. e0169648.
 34. Fagone, P., et al., *Systemic errors in quantitative polymerase chain reaction titration of self-complementary adeno-associated viral vectors and improved alternative methods*. Hum Gene Ther Methods, 2012. **23**(1): p. 1-7.
 35. Chou, H.H., et al., *Age-related immune clearance of hepatitis B virus infection requires the establishment of gut microbiota*. Proc Natl Acad Sci U S A, 2015. **112**(7): p. 2175-80.
 36. Durantel, D. and F. Zoulim, *New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus*. J Hepatol, 2016. **64**(1 Suppl): p. S117-31.
 37. Guidotti, L.G., et al., *High-level hepatitis B virus replication in transgenic mice*. J Virol, 1995. **69**(10): p. 6158-69.

661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720

38. Suarez-Amaran, L., et al., *A new HDV mouse model identifies mitochondrial antiviral signaling protein (MAVS) as a key player in IFN-beta induction.* J Hepatol, 2017.
39. Bian, Y., et al., *Vaccines Targeting PreS1 Domain Overcome Immune Tolerance in HBV Carrier Mice.* Hepatology, 2017.
40. Martin, P., et al., *TG1050, an immunotherapeutic to treat chronic hepatitis B, induces robust T cells and exerts an antiviral effect in HBV-persistent mice.* Gut, 2015. **64**(12): p. 1961-71.

721
722
723 **Figure legends**
724
725
726
727









728 **Figure 1: Detection of the HBV cccDNA in AAV-HBV transduced C57BL6 mice.** (A) Schematic
729 representation of HBV DNA forms extracted by a Hirt procedure and their expected
730 modifications after XhoI digestion. (B) Intrahepatic DNAs from an HBV-infected HuHep
731 mouse (day 84 p.i.), an AAV-HBV-transduced C57BL6/J mouse (day 28 p.t.) or HBV-infected
732 HepG2-NTCP cells (day 10 p.i.) were extracted following a Hirt procedure and submitted to
733 Southern blot analyses using HBV-DIG or AAV-DIG labeled probes. (C) Intrahepatic DNAs
734 from 20 AAV-HBV-transduced C57BL6/N mice (day 84 p.t.) were extracted following an Hirt
735 procedure, pooled in four different groups (#1, #2, #3, #4), digested or not by XhoI and
736 subjected to Southern blot analyses using HBV-DIG or AAV-DIG labeled probes. ND: not
737 digested, MW: molecular weight.
738
739
740
741
742
743
744
745
746
747
748
749
750

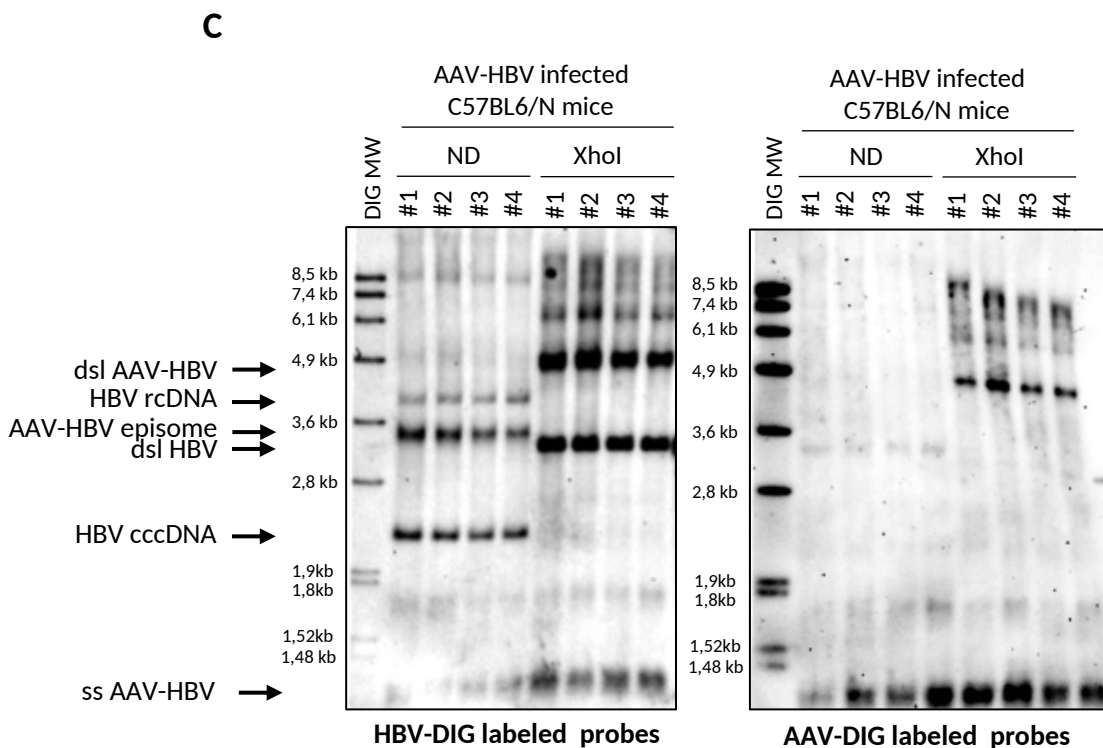
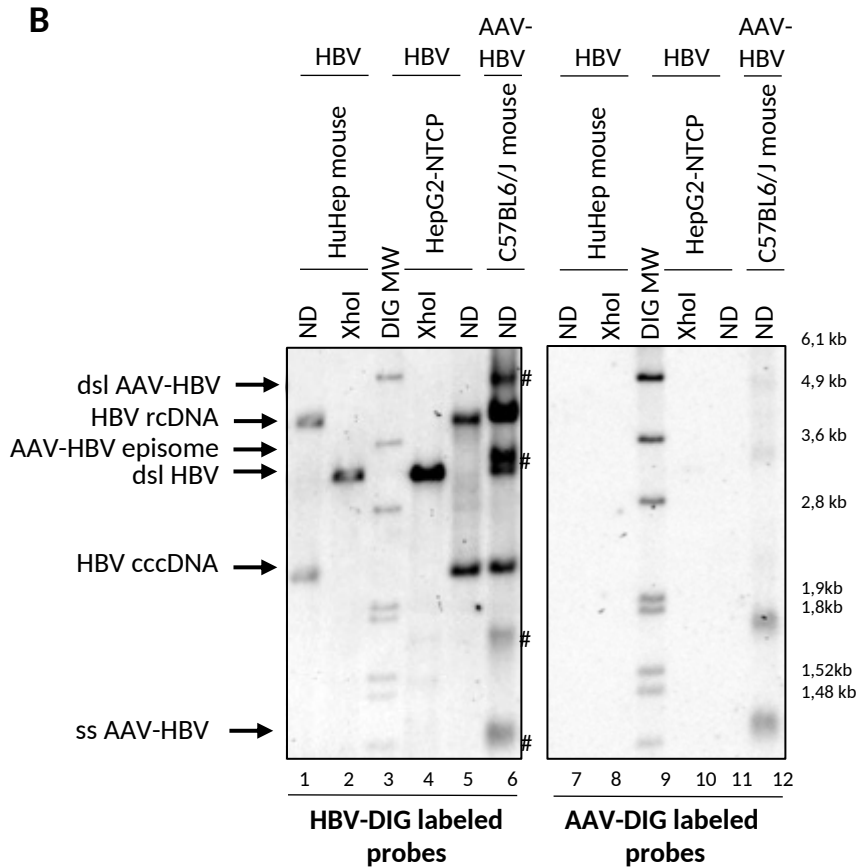
751
752 **Figure 2: Strategy to distinguish between the HBV cccDNA and the AAV-HBV episome in**
753 **AAV-HBV transduced C57BL6 mice.** (A) Schematic representation of HBV DNA forms
754 extracted by a Hirt procedure and their expected behavior after the indicated digestions. (B)
755 Intrahepatic DNAs from an AAV-HBV-transduced C57BL6/N mouse (day 28 p.t.) or HBV-
756 infected HepG2-NTCP cells (day 10 p.i.) were extracted following a Hirt procedure, digested
757 or not by indicated restriction enzymes and subjected to Southern blot analyses using HBV-
758 DIG or AAV-DIG labeled probes. (C) Intrahepatic DNAs from two AAV-HBV-transduced
759 C57BL6/J mice sacrificed respectively at day 21 p.t and day 28 p.t or HBV-infected HepG2-
760 NTCP cells (day 10 p.i.) were extracted following a Hirt procedure, digested or not by XmaI,
761 SacI and T5 and subjected to Southern blot analyses using HBV-DIG, AAV-DIG labeled probes
762 (left panels) or specific qPCR analyses to quantify total HBV DNA or cccDNA (right panels).
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780

781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840

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

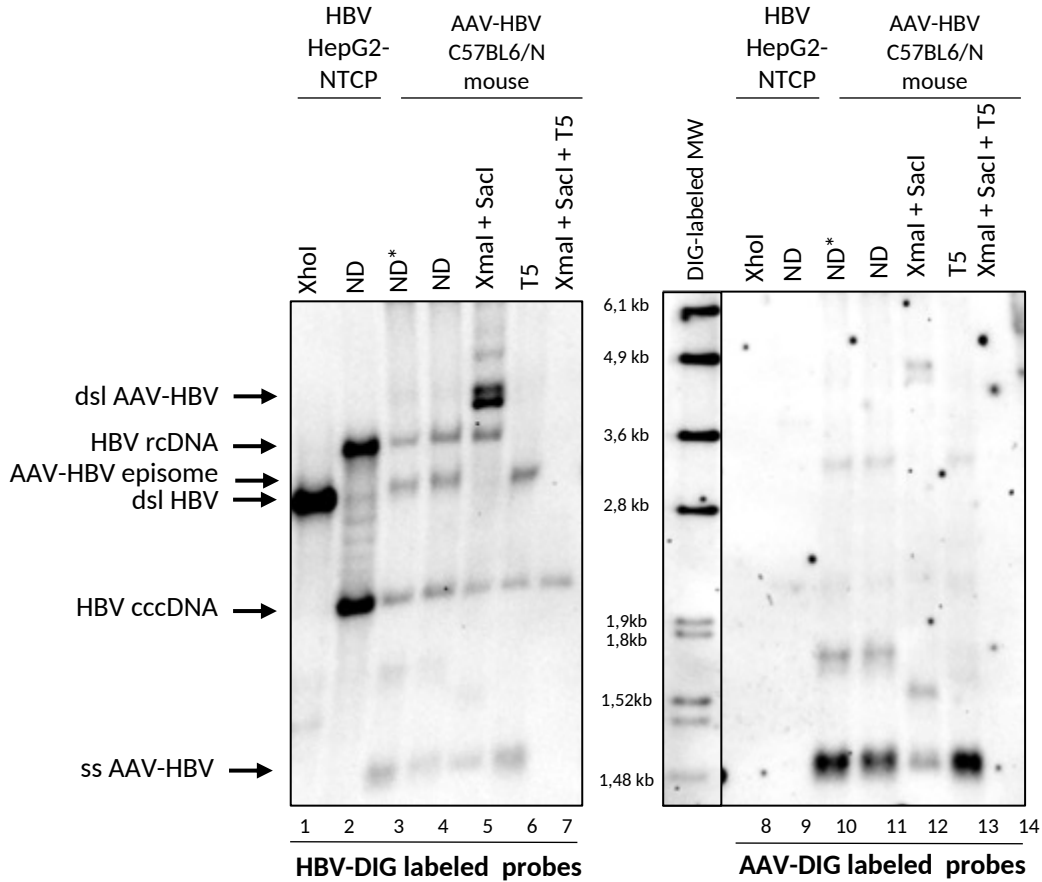
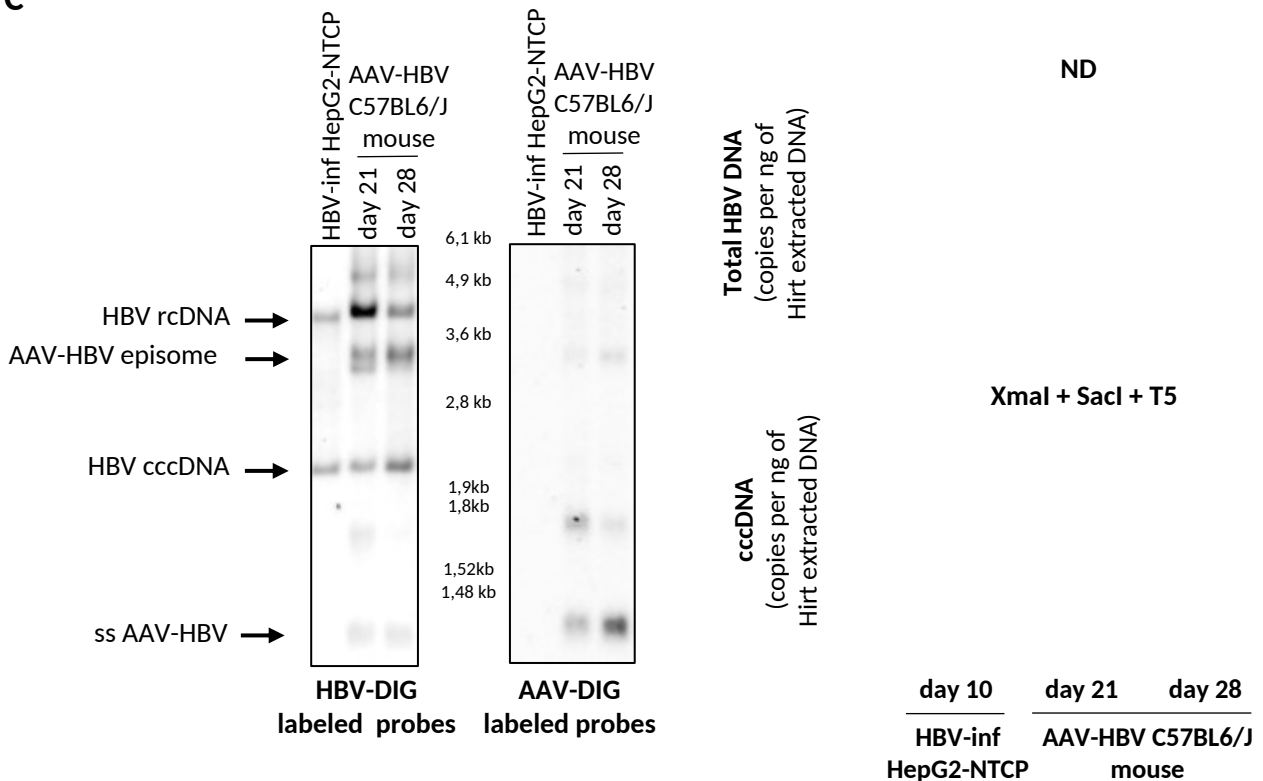
A

	no digestion (ND)	XhoI digestion
HBV rcDNA		 dsl HBV (3,2 kb)
HBV cccDNA	 supercoiled	 dsl HBV (3,2 kb)
AAV-HBV episome	 supercoiled	 dsl AAV-HBV (4,8 kb)
ss AAV-HBV		



A

	no digestion (ND)	T5 digestion	SmaI + SacI digestion	SmaI + SacI+ T5 digestion
HBV rcDNA		-		-
HBV cccDNA				
AAV-HBV episome				-
ss AAV-HBV		-		-

B**C**

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 Maily³, Barbara Testoni¹, Floriane Fusil⁴, Aurore Inchauspé^{1,2}, Maud Michelet¹, Marie-Louise Michel⁵, Massimo Levrero¹, Pierre Cortez², Thomas F. Baumert^{3,6}, François-Loïc 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 Hépatologie-digestif, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

⁷Hospices Civils de Lyon (HCL), Lyon, 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 1×10^{10} 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 10^5 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 $5 \cdot 10^{10}$ 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)).

University of Strasbourg's site. 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 10^{11} 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)).

CIRI's site. 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 5×10^8 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	ATGTGGTATTGGGGCCAAG
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	TGCGAATCCCACTCCGAAA
HBV	HBV-F8	AGACGAAGGTCTCAATCGCC
HBV	HBV-R8	ACCCACAAAATGAGGCGCTA
AAV	AAV.D-F1	CTCCATCACTAGGGGTTCTT
AAV	AAV-R1	CAATTCGCCCTATAGTGAGT
AAV	AAV-R2	GTTTCGAAATCGATAAGCTTGG

Quantitative PCR analyses. One microgram of Hirt extracted DNA were digested or not for 2h at 37°C with 2,5 U of XmaI (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

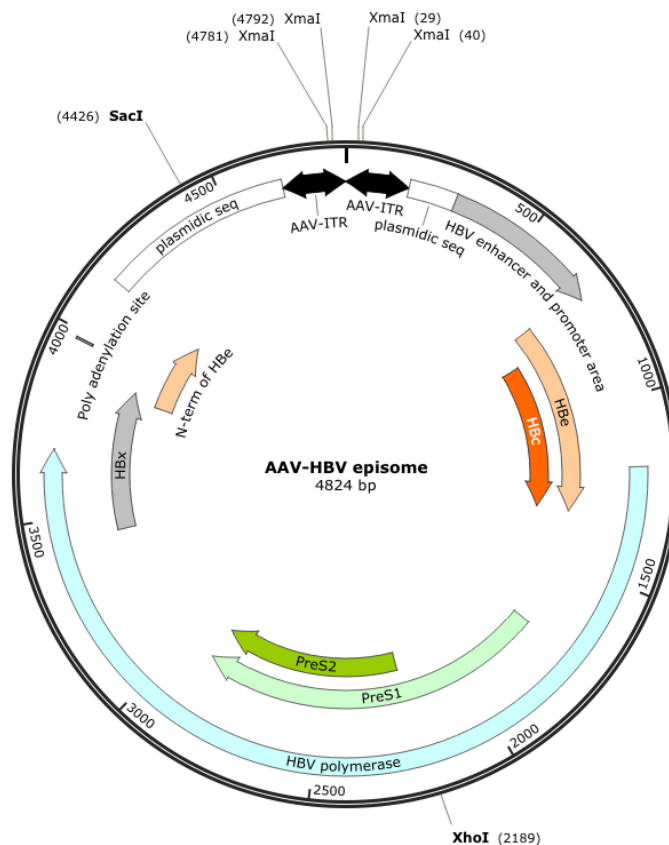
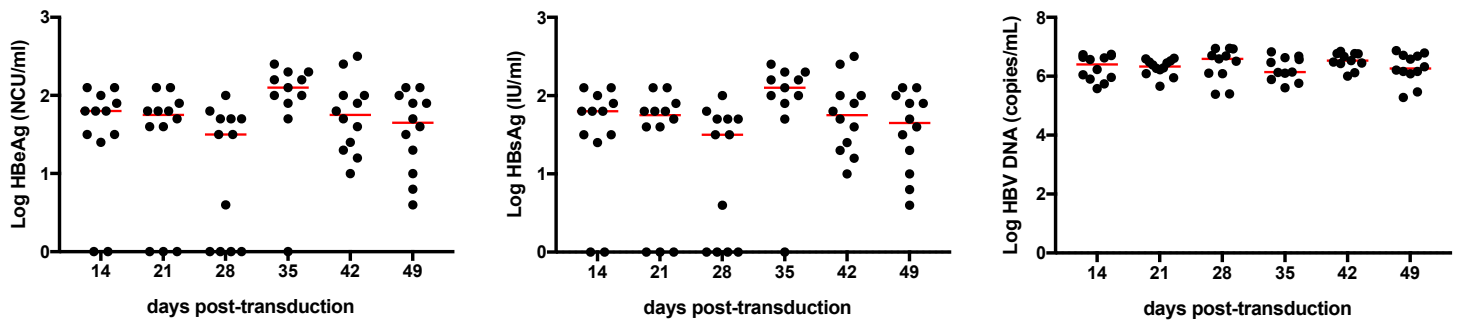


Figure S2



Twelve C57BL6/J mice received a single tail vein injection of $5 \cdot 10^{10}$ 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.