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

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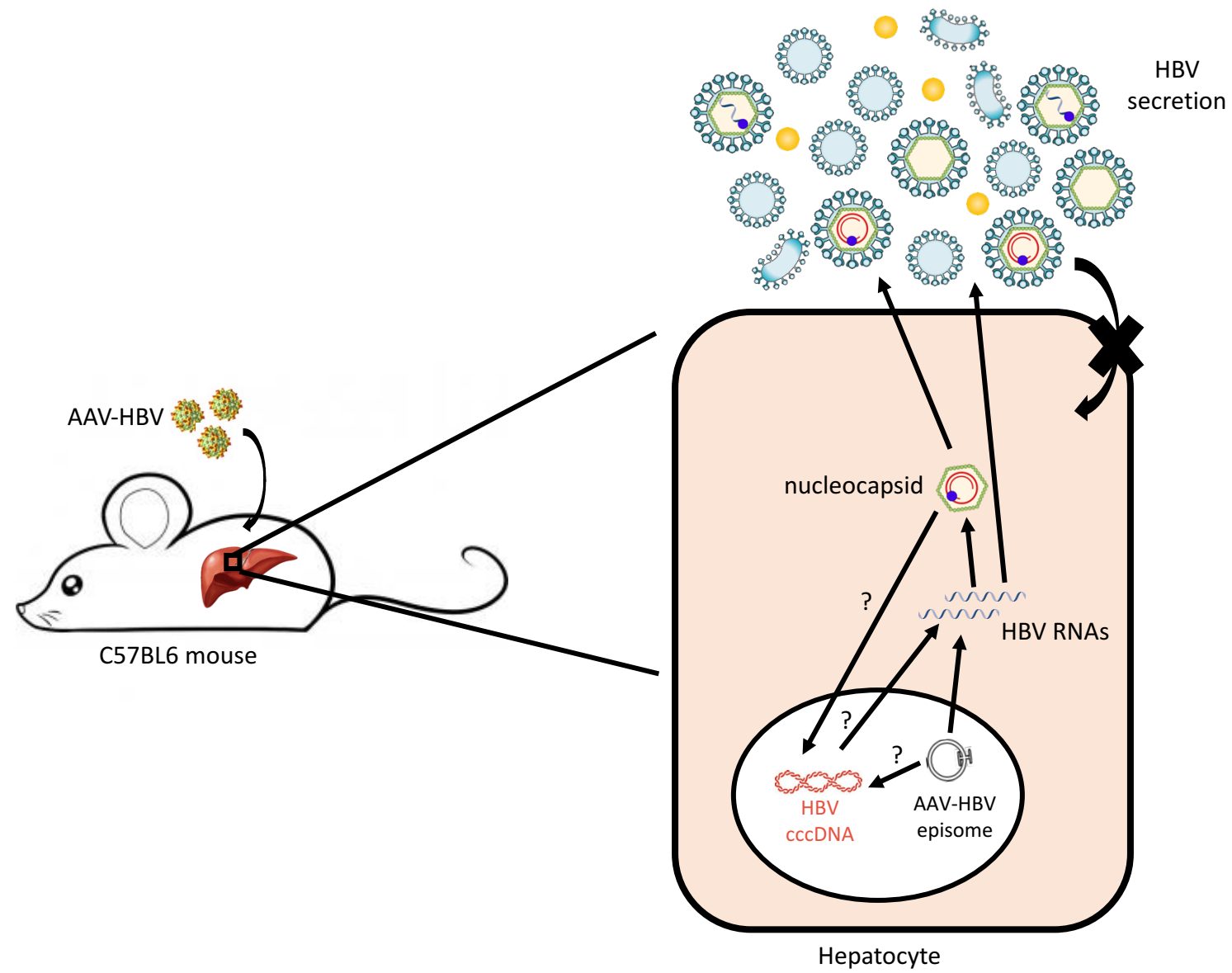
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Abbreviations

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*

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62
63 **Abstract**
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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,
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68 HBV cccDNA is a key replication intermediate in the viral life cycle. Little is known about the
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70 mechanisms involved in its formation, maintenance and fate under antiviral therapies. This is
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72 mainly due to the lack of small immune-competent animal models able to recapitulate the
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74 entire HBV replication cycle, including formation of HBV cccDNA. Here we report that HBV
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76 cccDNA can be detected by Southern blot analyses in the liver of C57BL6 mice transduced
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78 with AAV-HBV. HBV cccDNA persists in the liver of these animals together with the AAV-HBV
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80 episome. We also set up a PCR strategy to distinguish the HBV cccDNA from the AAV-HBV
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82 episome. These suggest that the AAV-HBV/mouse model might be relevant to test drugs
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84 targeting HBV cccDNA regulation and persistence.
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92 **Keywords**
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94 Hepatitis B virus, cccDNA, adeno-associated virus, immune-competent mouse, immune-
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96 therapeutics
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100 **Highlights**
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- 102 • The HBV cccDNA was detected by Southern blotting in AAV-HBV transduced C57BL6
103 mice
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- 105 • A strategy was set up to distinguish the HBV cccDNA from the AAV-HBV episome by
106 qPCR
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- 108 • AAV-HBV transduced immune-competent mice will allow to test drugs targeting
109 cccDNA regulation and stability *in vivo*
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123 **Main text**
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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].
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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
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183 available for experimental studies [14]. The use of Macaques, relevant for toxicology studies,
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185 is under investigation as an alternative *in vivo* model of HBV infection but economic reasons
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187 retrain their use [15].
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190 Mice, considered as a less expensive alternative animal model, are naturally not susceptible
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192 to HBV infection since the mouse sodium taurocholate cotransporting polypeptide (NTCP)
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194 orthologue does not allow HBV entry into murine hepatocytes [16, 17]. Mice or murine cell
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196 lines over-expressing the human NTCP can efficiently be infected by Hepatitis Delta virus
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198 (HDV) particles that share the same envelop as HBV and therefore enter by similar pathway.
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201 However, HBV replication was not detected and HBV cccDNA formation was suggested to be
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203 restricted in mouse cells [17-20]. To circumvent this issue, different alternatives have been
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205 proposed. Immune-deficient mice have been used to generate humanized liver models
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207 (HuHep mice) that are susceptible to HBV infection [21]. However, the absence of a
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209 functional immune system in these animals prevents the study of immunological issues
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211 regarding HBV infection, as well as the evaluation of novel immune-therapies. The injection
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213 of HBV minicircle or viral vectors in immune-competent mice was proposed to bypass
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215 limiting steps (i.e., entry and the HBV cccDNA formation) and allow transcription of HBV
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217 RNAs and virus production. Indeed, transfection of HBV minicircle led to the formation of
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219 HBV cccDNA-like molecules in hepatocytes and to persistent HBV replication *in vivo* [22, 23].
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223 Chronic HBV infection has also been successfully established in immune-competent mice by
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225 inoculating low doses of adenovirus- [24] or adeno-associated virus (AAV) vectors containing
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227 the HBV genome [25-27]. These models have proven useful for immunological studies.
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231 However, despite the fact that HBV cccDNA has been previously detected in HNF1a null HBV
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233 transgenic mice [28] and in a murine hepatic cell line derived from a hTGF-alpha transgenic
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235 mouse that harbor an inducible HBV genome integration [29], it was assumed that its
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243 formation and maintenance would not occur in AAV-HBV-transduced mice, as it was
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245 reported for humanized NTCP transgenic mice [17].
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250 Here, we investigated the establishment of an HBV cccDNA pool in HBV AAV8-HBV-
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252 transduced C57BL6 mice in comparison with HBV-infected HuHep mice and HBV-infected
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254 HepG2-NTCP cells. Intrahepatic DNA was extracted following a Hirt procedure that favors
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256 the enrichment of low molecular weight DNA such as the HBV cccDNA (see supplementary
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258 material and methods). It was then subjected to Southern blot analysis, the gold standard
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260 method to specifically detect HBV cccDNA [30]. Different HBV DNA forms were theoretically
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262 expected to be detected in AAV-HBV transduced cells. Those include the HBV polymerase-
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264 free rcDNA, the single-stranded (ss) AAV-HBV DNA (from incoming AAV particles), the
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266 episomal circular double-stranded (ds) AAV-HBV DNA monomers and multimers (formed by
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268 recombination, [31, 32]) as well as, hypothetically, the HBV cccDNA formed by re-import of
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270 HBV rcDNA from neo-formed cytoplasmic nucleocapsids (**Figure 1A**). The HBV DNA circular
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272 forms should theoretically all be linearized upon digestion at the unique XhoI site (**Figure**
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274 **1A**). HBV rcDNA and cccDNA were detected at their respective expected size (given their
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276 agarose mobility properties according to their relaxed or supercoiled state) in HBV-infected-
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278 HuHep mice and -HepG2-NTCP cells (**Figure 1B, lanes 1 and 5**). As expected (**Figure 1A**), XhoI
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280 digestion resulted in a single 3,2 kb band corresponding to a double stranded linear (DSL)
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282 HBV DNA (**Figure 1B, lanes 2 and 4**). Interestingly, we also observed signals corresponding to
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284 the HBV rcDNA and cccDNA forms in intrahepatic DNA extracted from an AAV-HBV-
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286 transduced C57BL6 mouse (**Figure 1B, lane 6**). Importantly, these forms were not detected
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288 with a specific DIG-labeled AAV-vector probes (**Figure 1B, lane 12**). Additional bands with
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290 mobility properties around 1.4 kb, 1.7 kb, 3.4 kb, and 4.8 kb (**Figure 1B, lanes 6, #**) were
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303 observed in the AAV-HBV-transduced C57BL6 mouse sample but not in the HBV-infected-
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305 HepG2-NTCP or -HuHep mouse samples. These additional bands corresponded to AAV
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307 sequences as confirmed by hybridization with specific AAV DIG-labeled probes (**Figure 1B,**
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309 **lane 12**). The 4.8 kb and 1.4 kb bands corresponded to the AAV-HBV dsDNA and ssDNA,
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311 respectively (**Figure 1A**). The two intermediate bands migrating at a 3.4 and 1.7 kb position,
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313 probably corresponded to circular episomal AAV-HBV monomers containing either a full-
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315 length or a truncated AAV-HBV genome, respectively. Detection of all these HBV DNA forms,
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317 including cccDNA, was confirmed in different AAV-HBV-transduced C57BL6 mice (coming
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319 from different laboratories but transduced with the same AAV8-HBV vector [25]) (**Figure 1C**).
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321 Digestion with an HBV single cutter mainly resulted in a 3.2 kb band corresponding to the
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323 HBV dsDNA and in a 4.8 kb band corresponding to the AAV-HBV dsDNA, while leaving AAV-
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325 HBV ssDNA intact (**Figure 1C, XhoI**). The higher MW forms visible above the AAV-HBV
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327 dsDNA after digestion with XhoI were most likely AAV-HBV concatemers.
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332 While Southern blot analyses allow to distinguish between the different HBV DNA forms, the
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334 sensitivity of the technique is rather low (around $7,5 \times 10^4$ copies with our method), time
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336 consuming and less reproducible than selective qPCR methods. In addition, if true
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338 discrimination of the HBV cccDNA from the almost identical viral linear DNA or rcDNA is still
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340 challenging, discrimination of HBV cccDNA from the AAV-HBV episome is even more
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342 challenging, as they not only share common sequences but also are both episomal forms. To
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344 increase the specificity of HBV cccDNA detection, a nuclease digestion is usually performed
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346 before selective qPCR methods based on the use of primers (and probes) spanning the nick
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348 in the HBV rcDNA and hybridizing to its "gap region". T5 exonuclease, that degrades HBV
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350 rcDNA but should leave episomal HBV cccDNA molecules intact, is currently widely used
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352 [33]. Accordingly, the cccDNA band was still detected in AAV-HBV samples after digestion
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363 with T5 (Fig.2B, lane 6). In addition, as expected, the circular closed AAV-HBV episome was
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365 also resistant to the T5 exonuclease activity (**Figure 2A, and 2B lanes 6 and 13**). Surprisingly,
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367 the AAV-HBV ssDNA band was also unaffected by T5 digestion (**Figure 2B, lanes 6 and 13**).
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369 This latter might be due to the complex secondary structure formed by the AAV ITRs that
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371 have been shown to be less accessible for DNA polymerases for instance [34]. To specifically
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373 detect only HBV cccDNA, we used a combination of digestion with XmaI and SacI restriction
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375 enzymes, that cut only in the AAV-HBV vector (**Figure S1, 2A, and 2B lanes 5 and 12**),
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377 followed by incubation with the T5 exonuclease resulting in complete degradation of all the
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379 AAV DNA species, including HBV-AAV SS forms (likely due to the cut in the ITRs by XmaI)
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381 (**Figure 2A, 2B lanes 7 and 14**). We confirmed that this triple digestion allowed to detect
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383 cccDNA by qPCR by comparing three different DNA samples (one extracted from HBV-
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385 infected HepG2-NTCP cells and two extracted from AAV-HBV-transduced C57BL6 mouse
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387 livers) containing different amounts of total HBV DNA and cccDNA (**Figure 2C**).

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395 Overall, we demonstrated that an HBV 3.2 kb cccDNA can be genuinely detected in livers
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397 from AAV-HBV-transduced C57BL6 mice that display a chronic HBV infection (as shown by
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399 stable secretions of HBeAg, HBsAg and viremia (**Figure S2**)). Until now, HBV cccDNA
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401 detection in mouse cells was reported in only two studies using HNF1a null HBV transgenic
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403 mice [28] or a murine hepatic cell line derived from a hTGF-alpha transgenic mouse that
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405 harbors an inducible and integrated HBV genome [29]. The advantage of the AAV-HBV-
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407 transduced mouse model over those latter models is that (i) different HBV genotypes or
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409 mutants can be easily inserted into the AAV vectors and (ii) different mouse strains with
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411 different genetic background can be used. The model will further allow studies to determine
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413 the half-life of HBV cccDNA and to understand if the presence of HBV cccDNA may influence
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423 and explain the outcome of the infection after AAV-HBV transduction in different mouse
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425 strains [35]. As the mouse NTCP orthologue does not allow HBV entry into cells [17], HBV
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427 propagation cannot occur in AAV-HBV transduced C57BL6 mice. This suggests that HBV
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429 cccDNA might be formed by recycling of *de novo*-formed cytoplasmic nucleocapsids in AAV-
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431 HBV transduced mouse hepatocytes. If so, the model would allow to (i) identify the factors
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433 that may influence HBV cccDNA formation by recycling of newly-formed cytoplasmic
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435 nucleocapsids (ii) to study the impact of nucleos(t)ides analogues or core protein allosteric
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437 modulators [36] on HBV cccDNA recycling within chronically infected hepatocytes and
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439 determine whether a combination of these two classes of antiviral agents could help
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441 depleting the pool of intrahepatic HBV cccDNA [11]. [Alternatively, HBV cccDNA could also be](#)
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443 [formed by recombination from the AAV-HBV episome. This could explain, at least in part,](#)
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445 [why HBV cccDNA was not detected in HBV-transgenic C57BL6 mice \[37\]. But this remains to](#)
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447 [be adequately investigated by long-term studies using nucleoside analogues to prevent](#)
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449 [potential recycling and/or AAV-HBV vector with mutation on ATGs of HBc gene to altogether](#)
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451 [prevent nucleocapsid formation. If cccDNA originates from genuine recycling the model will](#)
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453 [be useful to study both recycling itself and molecules, which could interfere with it. If](#)
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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](#)
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459 [the detected cccDNA is functional and can genuinely lead to viral RNA synthesis, as well as if](#)
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461 [it would eventually take over the AAV-HBV episome as the main template for viral](#)
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463 [transcription. These hypotheses are so far indirectly supported by a recent study describing](#)
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465 [the disappearance of AAV-HBV and AAV-HDV episomes in transduced C57BL6 mice without](#)
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467 [a significant decline of HBV viral load \[38\].](#)
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483 In conclusion, our results highlight the relevance of the AAV-HBV/mouse model that will be
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485 pivotal to decipher the mechanisms of HBV cccDNA maintenance and regulation as well as to
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487 evaluate the fate of HBV cccDNA under novel therapies combining direct acting antivirals
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489 with immune-modulatory drugs for instance [39, 40].
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495
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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)
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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
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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.

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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.

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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.

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723 **Figure legends**
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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.
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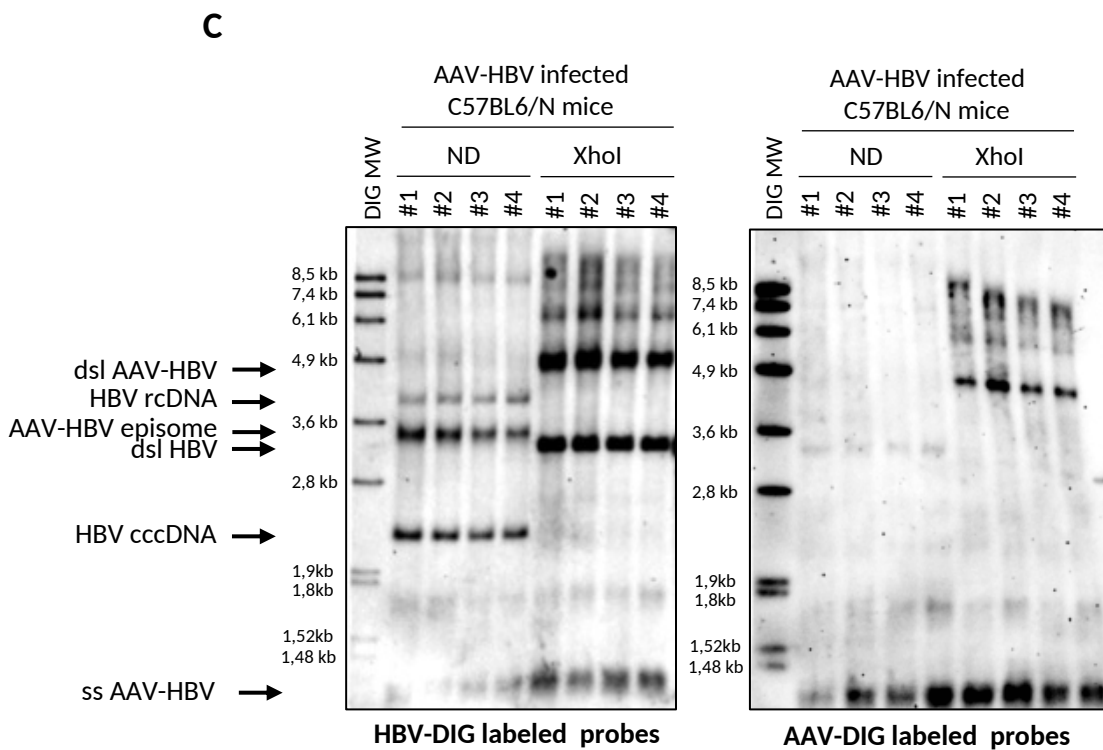
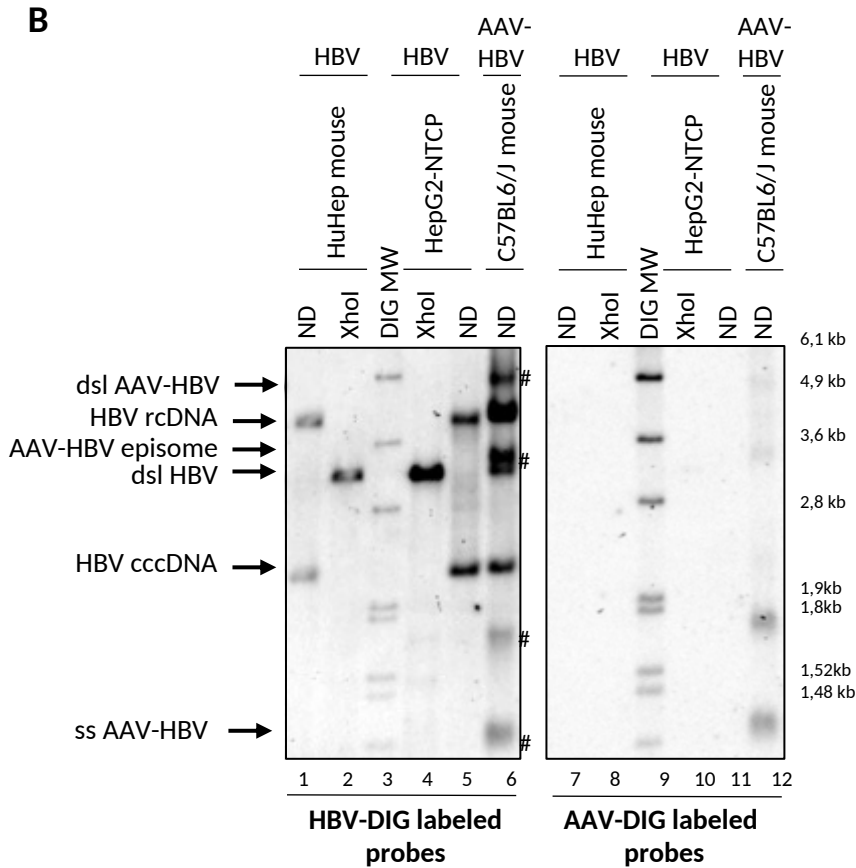
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752 **Figure 2: Strategy to distinguish between the HBV cccDNA and the AAV-HBV episome in**
753 **AAV-HBV transduced C57BL6 mice.** (A) Schematic representation of HBV DNA forms
754 extracted by a Hirt procedure and their expected behavior after the 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).
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ND: not digested, MW: molecular weight. *not digested with no digestion buffer or incubation at 37°C.

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	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		



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

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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	TGCGAATCCACACTCCGAAA
HBV	HBV-F8	AGACGAAGGTCTCAATCGCC
HBV	HBV-R8	ACCCACAAAATGAGGCGCTA
AAV	AAV.D-F1	CTCCATCACTAGGGGTTCT
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 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

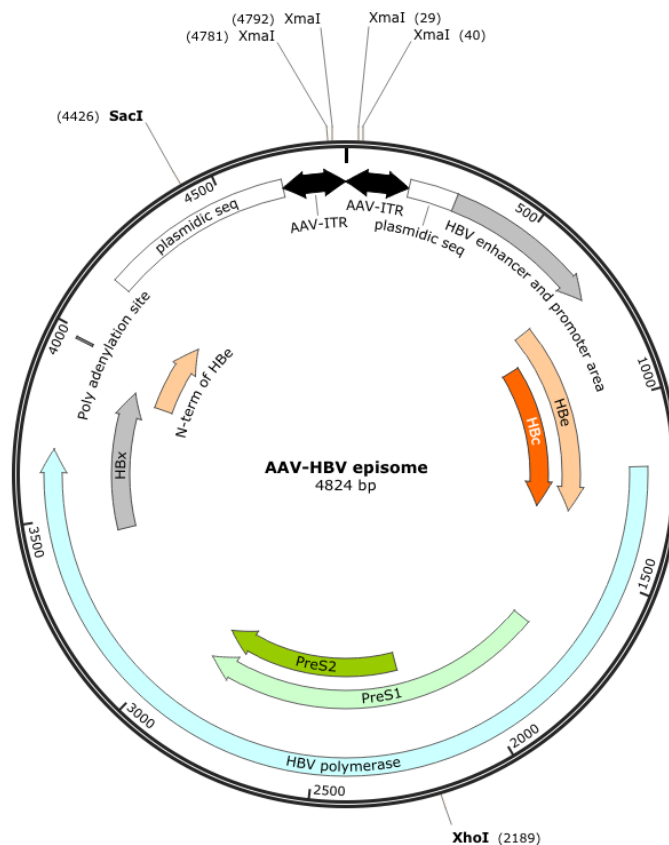
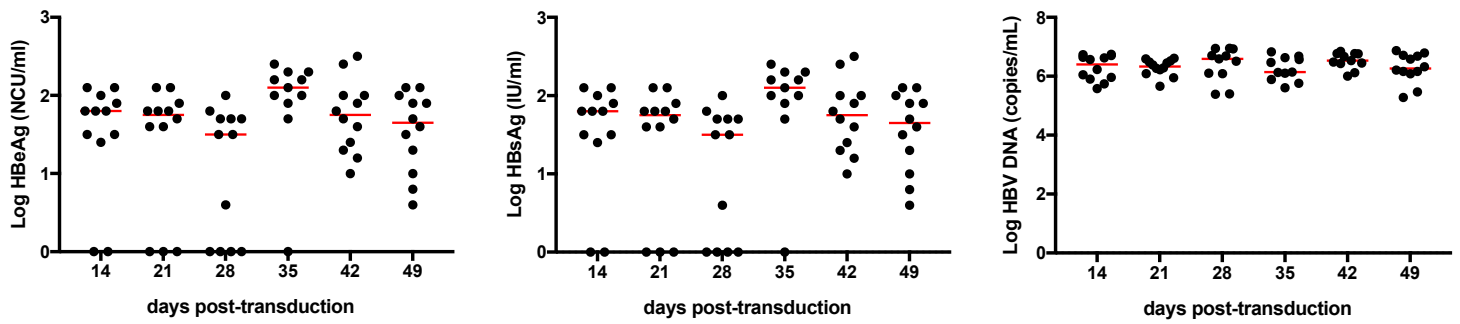


Figure S2



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.