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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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ABSTRACT

Hepatitis B Virus (HBV) persists in infected hepatocytes as an episomal covalently-closed-circular 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.

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 (Newbold et al., 1995; Bock et al., 1994). Host histone proteins as well as other proteins involved in gene expression regulation are bound to HBV cccDNA (Bock et al., 2001) and its transcriptional activity is subjected to the "histone code" (Pollicino et al., 2006). 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 (Seeger et al., 2013). The universal rebound of HBV replication upon withdrawal from nucleos(t)ide analogue

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.

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treatment (Zoulim and Locarnini, 2009), as well as traces of HBV-DNA remaining detectable years after clinical recovery from acute hepatitis (Rehermann et al., 1996; Maynard et al., 2005), 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 (Marcellin et al., 2003; Zoulim et al., 2016), but do not affect HBV cccDNA transcription template (Boyd et al., 2016).

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 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 (Guidotti et al., 1999; Wieland et al., 2004) but are no longer available for experimental studies (Harrington, 2012). 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 (Dupinay et al., 2013).

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 (Yan et al., 2012; Lempp et al., 2016). 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 (Lempp et al., 2016, 2017; He et al., 2015; Mailly et al., 2017). 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 (Allweiss and Dandri, 2016). 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 HBV cccDNA formation) and allow transcription of HBV RNAs as well as 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* (Guo et al., 2016; Yan et al., 2017). Chronic HBV infection has also been successfully established in immune-competent mice by inoculating low doses of adenovirus- (Huang et al., 2012) or adeno-associated virus (AAV) vectors containing the HBV genome (Dion et al., 2013; Yang et al., 2014; Ye et al., 2015). 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 (Raney et al., 2001) and in a murine hepatic cell line derived from a hTGF-alpha transgenic mouse that harbor an inducible HBV genome integration (Cui et al., 2015), 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 (Lempp et al., 2016).

Here, we investigated the establishment of an HBV cccDNA pool in AAV8-HBV-transduced 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 (Nassal, 2015). Different HBV DNA forms were theoretically expected to be detected in AAV-HBV transduced cells. Those include the HBV polymerase-free 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 (Nakai et al., 2000, 2001)) as well as, hypothetically, the HBV cccDNA formed by re-import of HBV rcDNA from neo-formed cytoplasmic nucleocapsids (Fig. 1A). The HBV DNA circular forms should theoretically all be linearized upon digestion at the unique *Xho*I site (Fig. 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 or HepG2-NTCP cells (Fig. 1B, lanes 1 and 5). As expected (Fig. 1A), *Xho*I digestion resulted in a single 3,2 kb band corresponding to a double stranded linear (dsl) HBV DNA (Fig. 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-HBV-transduced C57BL6 mouse (Fig. 1B, lane 6). Importantly, these forms were not detected with a specific DIG-labeled AAV-vector probes (Fig. 1B, lane 12). Additional bands with mobility properties around 1.4 kb, 1.7 kb, 3.4 kb, and 4.8 kb (Fig. 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 (Fig. 1B, lane 12). The 4.8 kb and 1.4 kb bands corresponded to the AAV-HBV dslDNA and ssDNA, respectively (Fig. 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 full-length 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 (Dion et al., 2013)) (Fig. 1C). Digestion with an HBV single cutter mainly resulted in a 3.2 kb band corresponding to the HBV dslDNA and in a 4.8 kb band corresponding to the AAV-HBV dslDNA, while leaving AAV-HBV ssDNA intact (Fig. 1C, *Xho*I). The higher MW forms visible above the AAV-HBV dslDNA after digestion with *Xho*I 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 $7{,}5 \times 10^4$ 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 (Niu et al., 2017). Accordingly, the cccDNA band was still detected in AAV-HBV samples after digestion with T5 (Fig. 2B, lane 6). In addition, as expected, the circular closed AAV-HBV episome was also resistant to the T5 exonuclease activity (Fig. 2A and 2B lanes 6 and 13). Surprisingly, the AAV-HBV ssDNA band was also unaffected by T5 digestion (Fig. 2B, lanes 6 and 13). This 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 (Fagone et al., 2012). To specifically detect only HBV cccDNA, we used a combination of digestion with *Xma*I and *Sac*I restriction