



Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound

Jie Wang^{1,†}, Tao Shen^{1,†}, Xiangbo Huang^{1,†}, G. Renuka Kumar^{2,†}, Xiangmei Chen¹, Zhenzhen Zeng¹, Ruiyang Zhang¹, Ran Chen¹, Tong Li¹, Tianying Zhang³, Quan Yuan³, Pao-Chen Li², Qi Huang², Richard Colonna², Jidong Jia⁴, Jinlin Hou⁵, Malcolm A. McCrae⁶, Zhiliang Gao^{7,*}, Hong Ren^{8,*}, Ningshao Xia^{3,*}, Hui Zhuang¹, Fengmin Lu^{1,*}

¹State Key Laboratory of Natural and Biomimetic Drugs, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China; ²Assembly Biosciences, Inc., San Francisco, CA, USA; ³State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China; ⁴Clinical Epidemiology and EBM Unit, Beijing Friendship Hospital, Capital Medical University, China; ⁵Hepatology Unit and Key Laboratory for Organ Failure Research, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou, China; ⁶The Pirbright Institute, Pirbright, UK; ⁷Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong Province, China; ⁸Department of Infectious Diseases, Institute of Viral Hepatitis, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, China

Background & Aims: Hepatitis B virus (HBV) RNA in serum has recently been linked to efficacy and prognosis of chronic hepatitis B (CHB) treatment. This study explored the nature, origin, underlying mechanisms, and potential clinical significance of serum HBV RNA.

Methods: The levels of HBV DNA and RNA were determined in the supernatant of induced HepAD38, HBV-expressing HepG2.2.15 cells and primary human hepatocytes (PHH), and in the serum of transgenic mice and CHB patients. NP-40 and proteinase K treatment, sucrose density gradient centrifugation, electron microscopy, northern blot, multiple identification PCRs and 5' rapid-amplification of cDNA ends were performed to identify the nature of serum HBV RNA.

Results: Although significantly lower than HBV DNA levels, abundant HBV RNA was present in the serum of CHB patients. A series of experiments demonstrated that serum HBV RNA was pregenome RNA (pgRNA) and present in virus-like particles. HBV pgRNA virion levels increased after blocking the reverse transcription activity of HBV DNA polymerase, and decreased

after blocking the encapsidation of pgRNA. Furthermore, the presence of HBV pgRNA virion was associated with risk of viral rebound after discontinuation of nucleot(s)ide analogues (NAs) therapy in CHB patients.

Conclusions: Serum HBV RNA was confirmed to be pgRNA present in virus-like particles. HBV pgRNA virions were produced from encapsidated particles in which the pgRNA was non- or partially reverse transcribed. Clinically, HBV pgRNA virion might be a potential biomarker for monitoring safe discontinuation of NA-therapy.

Lay summary: HBV may have another virion form in which the nucleic acid is composed of RNA, not DNA. The level of HBV RNA virion in serum may be associated with risk of HBV viral rebound after withdrawal of treatment, and therefore, a potential predictive biomarker to monitor the safe discontinuation of nucleot(s)ide analogues-therapy.

© 2016 European Association for the Study of the Liver. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Keywords: Hepatitis B virus; HBV pgRNA virion; Nucleot(s)ide analogues therapy. Received 28 November 2015; received in revised form 20 May 2016; accepted 20 May 2016; available online 28 May 2016

* Corresponding authors. Address: State Key Laboratory of Natural and Biomimetic Drugs, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100191, PR China. Tel./fax: +86 10 82805136.

E-mail addresses: gaozl@21cn.com (Z. Gao), renhong0531@vip.sina.com (H. Ren), nsxia@xmu.edu.cn (N. Xia), lu.fengmin@hsc.pku.edu.cn (F. Lu).

[†] These authors contributed equally as joint first authors.

Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; pgRNA, pregenome RNA; NAs, nucleot(s)ide analogues; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; ETV, entecavir; LMV, lamivudine; EoT, end of treatment; ADV, adefovir dipivoxil; LdT, telbivudine.



ELSEVIER

Introduction

Hepatitis B virus (HBV) infection is a serious global public health problem with more than two billion people infected with HBV worldwide. It is estimated that every year ~786,000 people will die of chronic HBV infection-related diseases including liver cirrhosis or hepatocellular carcinoma (HCC) [1–3].

HBV belongs to the Hepadnaviridae family, which are defined as double-stranded DNA viruses replicating their DNA genome through reverse transcription from pre-genomic RNA (pgRNA).

During viral morphogenesis, pgRNA is encapsidated into core particles and reverse transcribed by HBV DNA polymerase into rcDNA. Mature rcDNA-containing virions are then enveloped and released from infected hepatocytes [4–6]. Importantly, in addition to HBV DNA, HBV RNA has also been detected in the serum of chronic hepatitis B (CHB) patient [7–14]. However, the life cycle of HBV cannot fully explain the generation of serum HBV RNA.

While nucleot(s)ide analogues (NAs) like entecavir (ETV) and tenofovir can significantly inhibit HBV replication and decrease serum HBV DNA to undetectable level, it is still difficult to achieve clinical cure of CHB due to the persistence of covalently closed circular DNA (cccDNA) in the nuclei of infected cells [15,16]. Once treatment is discontinued, a relapse in HBV levels is observed. Therefore, CHB patients require a long-term treatment. Unfortunately, long-term treatment with NAs possessing a low barrier to resistance, such as lamivudine, may lead to the appearance of drug-resistant viral mutants and subsequent risk of serious hepatitis flare-ups [17]. Thus, how and when to adjust the treatment regimen is a key point during the treatment of CHB. In this context, serum HBV RNA has been reported as an important potential predictor of the tyrosine-methionine-aspartate-aspartate (YMDD) resistance mutation [11], efficacy [9,12–14] and prognosis [10] of CHB treatment. Whether dynamic changes in serum HBV RNA can be used as a potential marker for the safe discontinuation of NAs-therapy remains an open question.

In this study, the nature and origin of serum HBV RNA was investigated in both *in vitro* and *in vivo* experiments to mechanistically determine the potential clinical significance of HBV RNA in serum.

Material and methods

Cell lines

The human liver cancer cell line HuH7 [18], the stable HBV-expressing human liver cancer cell lines HepG2.2.15 [19] and HepAD38 cells [20] were all maintained in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, Calif, USA).

Primary human hepatocytes (PHH)

PHH were kindly provided by Professor Hongkui Deng from the Department of Cell Biology, School of Basic Medical Sciences, Peking University Stem Cell Research Center. The cells were maintained in primary hepatocytes maintenance medium (PMM) as previously described [21].

Patient specimens

Patient cohort A included 11 Han Chinese CHB patients who had received telbivudine (TBV) therapy for more than 6 months at the Second Affiliated Hospital of Chongqing Medical University. The clinical background of the patients in cohort A is shown in Table 1. Patient cohort B included 33 Han Chinese CHB patients who had received NA-therapy for more than 3 years at the Third Affiliated Hospital of Sun Yat-Sen University and subsequently discontinued NA-therapy. The discontinuation of NA-therapy in all cases was decided by the attending physician with use of similar but not uniform criteria. The clinical background of the patients in cohort B is shown in Table 2. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. This study was approved by the Ethics Committee of Peking University Health Science Center. Written informed consent was obtained from each patient.

Table 1. Clinical background of the patient cohort A.

Characteristics	Values
Gender (M:F)	7:4
Age (years) ^a	39 (24-52)
ALT (IU/L) ^a	141 (65-318)
HBV DNA (log ₁₀ copies/ml) ^a	7.87 (6.56-9.01)
HBsAg (IU/ml) ^a	15,695 (1221-196,392)
HBeAg (S/CO) ^a	1846 (83-4528)
NAs	LdT
Duration of NAs therapy (weeks) ^a	236 (24-268)

^aMedian (range). NAs, nucleot(s)ide analogues; TBV, telbivudine.

Table 2. Clinical background of the patient cohort B.

Characteristics	DNA rebound (n = 24)	DNA non-rebound (n = 9)
Gender (M:F)	2:1	5:4
Age (years)	33 (18-54)	28 (23-59)
NAs	LMV/ETV/ADV/LdT	LMV/ETV/ADV/ LdT
End of treatment		
HBV DNA (log ₁₀ copies/ml)	below the LoD	below the LoD
HBsAg (IU/ml)	1503 (80.01-22,486)	329.8 (0.05-41,280)
HBeAg (S/CO)	0.120 (0.075-0.929)	0.112 (0.086-0.387)
HBeAb (S/CO)	0.024 (0.004-1.06)	0.007 (0.004-1.3)
AST (IU/L)	24 (14-44)	21 (15-28)
ALT (IU/L)	24 (11-45)	16 (6-21)
24 weeks post EoT		
HBV DNA (log ₁₀ copies/ml)	5.00 (2.26-8.35)	below the LoD
HBsAg (IU/ml)	2444 (74.4-19,280)	77.6 (0.05-2820)
HBeAg (S/CO)	0.101 (0.076-0.481)	0.141 (0.125-0.179)
HBeAb (S/CO)	0.004 (0.003-0.985)	0.92 (0.003-1.65)
AST (IU/L)	27 (16-330)	24 (18-30)
ALT (IU/L)	29 (6-610)	18 (13-23)

LMV, lamivudine, ETV, entecavir, ADV, adefovir dipivoxil, LdT, telbivudine, LoD, lower limit of detection.

Transgenic mice

The HBV transgenic (HBV-Tg) mice were provided by Pei-Jer Chen from the Department of Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine [22]. All mice were maintained under specific pathogen-free conditions in the Laboratory Animal Center of Xiamen University. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Compounds

Entecavir (ETV) crude drug was kindly provided by Fujian Cosunter pharmaceutical company (Fuzhou, Fujian, China) or Acme Biosciences (Palo Alto, CA, USA), and was dissolved in N, N-Dimethylacetamide (Sigma-Aldrich, St. Louis, MO, USA) to generate a 100 mM stock solution and stored at –20 °C. GLS4 was synthesized by published procedures [23], and was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to generate a 2 mM to 100 mM stock solution and stored at –20 °C.

Research Article

Plasmids

The 1.2 x HBV construct (pBB4.5-HBV1.2, genotype C) was constructed using a 1.2-fold length genome of genotype C HBV DNA based on pBB4.5-HBV1.2 (genotype D, G1896A mutation) which was kindly provided by Professor Locarnini, from the Victorian Infectious Diseases Reference Laboratory, Australia [24]. A mutant plasmid carrying the Y63F mutation was generated from pBB4.5-HBV1.2 using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Primer sequences were: Forward: 5'-GTGGGAAACTTTACTGGCTTTTCTCTACTGTACC TATCTTAATCC-3'. Reverse: 5'-GGATTAAGATACAGTAGAAGAGAAAAGCCAGTA AAGTTTCCCAC-3'.

HBV infection

HBV infection of PHH was conducted as previously described [25]. Briefly, 1×10^5 PHH cells in a 48-well plate were inoculated with 1×10^7 copies of genome equivalent HBV in the presence of 4% PEG 8000 for 20 h. PHH cells were then washed with PBS six times and maintained in PMM medium with medium change every 2–3 days.

Extraction and reverse transcription of HBV RNA

HBV RNA was isolated using the EasyPure[®] Viral RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). Isolated HBV RNA was reverse transcribed using RevertAid[™] First Strand DNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) with either random primers or an HBV specific RT primer. The sequence of HBV specific RT primer was: 5'-ATTCTCAGACCGTAG CACACGACACCGAGATTGAGATCTTCTGCGAC-3' in which the random sequence ATTCTCAGACCGTAGCACACGACAC was anchored at the 5' end of HBV specific sequence CGAGATTGAGATCTTCTGCGAC (nucleotide [nt] 2436–2415) and used for detection of the 3.5 kb HBV RNA, or: 5'-ATTCTCAGACCGTAGCACACGAC CACCGCTCAAGGTCGGTCTGAC-3' in which the random sequence ATTCTCAG ACCGTAGCACACGACAC was anchored at the 5' end of HBV specific sequence GCCTCAAGGTCGGTCTGAC (nt 1702–1682) and used for detection of total HBV RNA.

Multiple identification PCRs

According to the difference of nucleotide sequence between preC mRNA and pgrNA, we designed preC mRNA and 3.5 kb HBV RNA-specific primers. The sequences of 3.5 kb RNA and preC mRNA specific forward primer were: F (nt 1856–1877): 5'-CCTACTGTCAAGCCTCCAAGC-3', and F (nt 1796–1813): 5'-GGTC TGTTACCAGCACC-3', respectively. Both of the reverse primer was the anchored random sequence: 5'-ATTCTCAGACCGTAGCACACGACAC-3'. PCR reaction mixture (20 μ l) contained 10 μ l 2 \times Taq mix (GenStar, Beijing, China), 1 μ l forward primer (10 μ M), 1 μ l reverse primer (10 μ M), 1 μ l cDNA template and 7 μ l double distilled water (ddH₂O). The reaction mixture was denatured at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and at last 72 °C for 7 min. DNA fragments of varying lengths were resolved by a 1.5% agarose gel.

Quantification of HBV RNA and HBV DNA

The levels of HBV RNA were detected by quantitative real-time polymerase chain reaction (qPCR) in LightCycler 480 II Real-time PCR Detection System (Roche, Mannheim, Germany) with a SYBR Green or TaqMan[®] probe method. The primers and probe used to detect HBV 3.5 kb RNA were as follows: F1 (nt 2295–2312): 5'-AYAGACCATCAAATGCC-3', R1 (Anchored sequence): 5'-ATTCTCAGACCGTAGCA CACGACAC-3' and probe: 5' FAM-CCTATCAACACTTCCGGARACTACTGTGTTA GAC-BHQ1-3'. The primers and probe used to detect HBV total RNA were as follows: F1 (nt 1554–1572): 5'-TGTGCTTCTCATCTGCGC-3', R1 (Anchored sequence): 5'-ATTCTCAGACCGTAGCACACGACAC-3' and probe: 5' FAM-CGTGTGC ACTTCGCTTCACTCTGC-BHQ1-3'. The standards were constructed by PCR using each primer from HepAD38 cDNA, and the PCR products were subsequently ligated into the pEASY[®]-Blunt Cloning Vector (TansGen, Beijing, China). The qPCR reaction mixture (30 μ l) contained 15 μ l 2 \times mix (LightCycler[®] 480 Probes Master, Roche, Mannheim, Germany), 1 μ l forward primer (10 μ M), 1 μ l reverse primer (10 μ M), 1 μ l TaqMan probe (10 μ M), 3 μ l cDNA template and 9 μ l double distilled water (ddH₂O). The reaction mixture was denatured at 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 40 s. The level of HBV DNA was quantified by the COBAS[®] TaqMan 48[®] assay (Roche).

Quantification of HBsAg and HBeAg

The level of HBsAg and HBeAg was measured by a time-resolved fluoroimmunoassay (TRFIA) as previously described [16] or by a commercial enzyme-linked immunosorbent assay (Wantai Biological Pharmacy Enterprise Co., Beijing, China).

PEG-precipitation

6 x PEG8000 buffer (48% PEG8000 and 200 mM NaCl) was added to the cellular lysate or supernatant of HepAD38 cells at a volume ratio of 1 to 5. The mixture was shaken slowly overnight at 4 °C, and then centrifuged at 7500 rpm (7232 g) with the brake "5" for 30 min at 10 °C in an Eppendorf HL019 rotor (Centrifuge 5810 R, Eppendorf, Germany). The PEG-precipitated pellet was digested with Micrococcal Nuclease (New England Biolabs (NEB), Ipswich, MA, USA) and proceeded for DNA/RNA extraction, Western Blot analysis, or sucrose density gradient centrifugation.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was performed as previously described [26]. Briefly, discontinuous sucrose density gradients (15, 25, 35, 45 and 60%) were prepared with a solution containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl and 1 mM EDTA. Culture supernatant was laid on the linear sucrose gradient, and centrifuged at 25,000 rpm (112,000 g) for 15 h at 10 °C in a Beckman SW50.1 rotor (Beckman Coulter, Fullerton, CA, USA).

Electron microscopy

For conventional transmission electron microscopic observation, a drop of fraction 26 separated by sucrose density gradient centrifugation was placed on a carbon-coated 200-mesh copper grid. The grid was negatively stained with phosphotungstic acid (1%, pH 6.8) for 1 min, and then examined by a transmission electron microscope (FEI-TECNAI12, New York, USA) at 80 kV.

Northern blot

TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA) was added to the virus pellet following PEG-precipitation, and total RNA was prepared in accordance to the manufacturer's instructions. RNA was mixed with denaturation buffer and heated at 65 °C for 10 min, and immediately transferred to an ice bath. Denatured samples were resolved on a 1.2% agarose gel in 1 x MOPS buffer at 70 V for 3 h. RNA in the gel was transferred to nylon membrane for 1.5–2 h by vacuum. After transfer, the membrane was UV cross-linked for 10 min. Pre-hybridization was performed in pre-warmed "DIG Easy Hyb buffer" at 50 °C for 2 h with gentle agitation. For hybridization, the membrane was gently agitated in pre-warmed "DIG Easy Hyb buffer" containing the DNA probe that had been denatured by boiling for 5 min. The membrane was washed by 2 x SSC/0.1% sodium dodecyl sulfate, and then 0.5 x saline sodium citrate/0.1%SDS at 60 °C and rinsed with 1 x maleic acid buffer for 5 min. The membrane was blocked in 1% blocking buffer for 30 min and incubated in anti-DIG antibody solution for 1 h, followed by rinsing with 1 x washing buffer and incubating in 1 x detection buffer for 5 min. Finally, the membrane was incubated with CDP-star[®] and exposed.

Western blot

PEG-precipitated pellets from HepAD38 cells or supernatant were dissolved in 1x Laemmli buffer (with 5% 2-mercaptoethanol), boiled for 10 min at 95 °C and resolved on 4–20% gradient SDS-PAGE gel (BioRad). HBV core protein was detected with anti-Core antibody (DAKO, Denmark, or a gift from State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, Fujian, China).

Rapid-amplification of cDNA ends (RACE)

HBV RNA in fraction 26 of sucrose density gradient centrifugation was isolated using the EasyPure Viral RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions, and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). RACE amplification was performed using the SMARTer[™] RACE cDNA Amplification Kit (Clontech, Laboratories, Inc., Mountain View,

CA, USA) according to the manufacturer's instructions. Briefly, the SMARTer first strand synthesis was performed using the SMARTer II A oligonucleotide (AAG CAGTGGTATCAACGCAGAGTACXXXXX; X = undisclosed base in the proprietary SMARTer ologo sequence). The sequence of HBV specific RT primer was 5'-CGA GATTGAGATCTTCTGCGAC-3'. The sequences of HBV specific reverse primers used for semi-nested PCR were: R1: 5'-GTTGATAAGATAGGGGCATTTGGTGGTC-3', and R2: 5'-GAAGGAAAGAAGTCAGAAGGCAA-3'. Both of forward primer was: 5'-CTAA TACGACTACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'.

Statistical analyses

The repeated measures analysis of variance and two groups of related non-parameters Wilcoxon symbols test were performed using the statistical software package SPSS version 21.0 for Windows (SPSS, Chicago, IL, USA). All tests of significance were two-tailed and $p < 0.05$ was considered statistically significant.

Results

Serum HBV RNA is pregenome RNA

We systematically investigated the nature of the serum-associated HBV RNA in order to better understand its clinical significance. First, the levels of HBV DNA and HBV RNA in sera of 11 treatment-naïve CHB patients (cohort A) were determined by quantitative real-time PCR (qPCR) or RT-PCR (RT-qPCR). The median baseline levels of HBV DNA and HBV RNA in serum were 7.87 (6.56–9.01) \log_{10} copies/ml and 6.31 (5.10–8.02) \log_{10} copies/ml, respectively. Thus, the presence of serum HBV RNA was demonstrated in the sera of CHB patients, though HBV RNA levels were significantly lower than HBV DNA levels ($p = 0.007$, Fig. 1A). Total HBV RNA and 3.5 kb HBV RNA levels in the sera of three treatment-naïve CHB patients were subsequently analyzed by RT-qPCR. The efficiency of RT-qPCR for 3.5 kb HBV RNA or total HBV RNA is shown in each standard curve (Supplementary Fig. 1). To rule out interference from residual HBV DNA, samples were treated with DNase I before reverse transcription was performed. The level of 3.5 kb HBV RNA was almost equal to that of total HBV RNA in all three patients, suggesting that 3.5 kb HBV RNA was the predominant type of HBV RNA present in serum (Fig. 1B). The qPCR results were confirmed by northern blot analysis, and demonstrated that only the 3.5 kb RNA could be detected in the supernatant of untreated HepAD38 cells (Fig. 1C). Collectively, these data indicate that the 3.5 kb RNA is the only HBV RNA present in patient sera.

The 3.5 kb HBV RNA represents both preC mRNA and pgRNA. In order to clearly identify the 3.5 kb HBV RNA detected in serum, we performed multiple identification PCRs. We used 3.5 kb HBV RNA-specific primers that amplify both preC mRNA and pgRNA and preC mRNA specific primers that only amplify preC mRNA (Fig. 1D). The discriminatory specificities of the designed primers were first confirmed with RNAs extracted from HBV producing cell lines including HepAD38 and HepG2.2.15 cells. As shown in Fig. 1E, the specific primers could efficiently amplify preC mRNA and 3.5 kb HBV RNA from cell lysates, respectively. Only the 3.5 kb HBV RNA, and not preC mRNA, was detected in the supernatants of either HepAD38 or HepG2.2.15 cells, even after treatment with ETV. ETV treatment resulted in a large increase in the level of 3.5 kb HBV RNA, which indicated that just pgRNA was present in the culture supernatant. Consistent with this observation, only pgRNA was detected in the sera of three treatment-naïve CHB patients, confirming that the HBV RNA detected in serum is pgRNA (Fig. 1F).

Serum HBV pgRNA is present in HBV-like viral particles

Several studies have reported that the HBV RNA detected in serum may be incorporated into HBV particles [9,12], although its precise form is poorly understood. In order to address this, HepAD38 cell supernatant was treated with NP-40 to remove the lipid membrane bound structures or with proteinase K to destroy the proteinaceous structure. HBV RNA was only detected in the supernatant treated with proteinase K, indicating that HBV RNA in HepAD38 supernatant was contained within a proteinaceous structure, most likely in the nucleocapsids (Fig. 2A). Consistent with previous observations (Fig. 1E), the level of HBV RNA detected following proteinase K treatment was much higher in the supernatant of HepAD38 cells treated with ETV as compared to untreated cells (Fig. 2A).

Supernatant of both untreated and ETV-treated HepAD38 cells was subjected to PEG-precipitation followed by sucrose density gradient centrifugation, with the levels of HBsAg, HBeAg and HBV DNA in each gradient fraction analyzed. As shown in Fig. 2B, HBV DNA peaked in fraction 26 for both untreated and ETV-treated samples. Similarly, HBeAg also peaked in fraction 26, suggesting that HBV viral particles were enriched in this fraction (Fig. 2C). The HBsAg was enriched in fractions 18–25, suggesting that HBV filaments and spheres were concentrated in these fractions (Supplementary Fig. 2). The HBV RNA had a similar fraction distribution as HBV DNA and HBeAg, suggesting that the HBV RNA detected in the supernatant of HepAD38 cells or in the serum of CHB patient was incorporated into HBV-like viral particles (Fig. 2B). To further confirm this observation, we measured the changes of HBV DNA, RNA and HBeAg levels in the supernatant of ETV-treated HepAD38 cells. We postulated that if HBV particles consist of only HBV DNA, then the levels of HBeAg should decline to the same degree as that of HBV DNA in ETV-treated HepAD38 cell supernatants. Results show that HBeAg levels did not change significantly, indicating that HBV particle's composition was not limited to HBV DNA (Fig. 2D; Supplementary Fig. 3). Parallel analysis of HBV DNA and HBV RNA revealed that while HBV DNA levels were dramatically reduced in HepAD38 supernatants in the presence of ETV ($5.94 \pm 0.03 \log_{10}$ copies/ml) compared to untreated cells ($7.01 \pm 0.04 \log_{10}$ copies/ml), the levels of HBV RNA increased more than 10-fold ($5.91 \pm 0.06 \log_{10}$ copies/ml vs. $7.00 \pm 0.03 \log_{10}$ copies/ml). Of particular note was the finding that HBV RNA levels in the ETV-treated HepAD38 cell supernatants ($7.00 \pm 0.03 \log_{10}$ copies/ml) was almost equal to that of HBV DNA levels in untreated HepAD38 cell supernatants ($7.01 \pm 0.04 \log_{10}$ copies/ml). These results combined with the observation that HBeAg levels remain unchanged in ETV-treated HepAD38 cell supernatants provide further evidence that HBV RNA is present in HBV-like viral particles (Fig. 2D).

Previous reports indicate that viral RNA can be present in exosomes [27,28]. In order to determine if our results are due to exosome-associated HBV RNA, we treated HepAD38 cells with GW4869, a known inhibitor of exosome secretion. GW4869 treatment reduced the expression of exosome-specific protein CD63 but did not affect the levels of HBV RNA (Fig. 2E), indicating that serum HBV RNA is not associated with exosomes. Additionally, electron microscopy of fraction 26 showed that similar HBV-like viral particles were present in both ETV-treated and untreated HepAD38 cell supernatants (Fig. 2F). Given that the levels of HBV DNA and RNA are different in these samples, this

Research Article

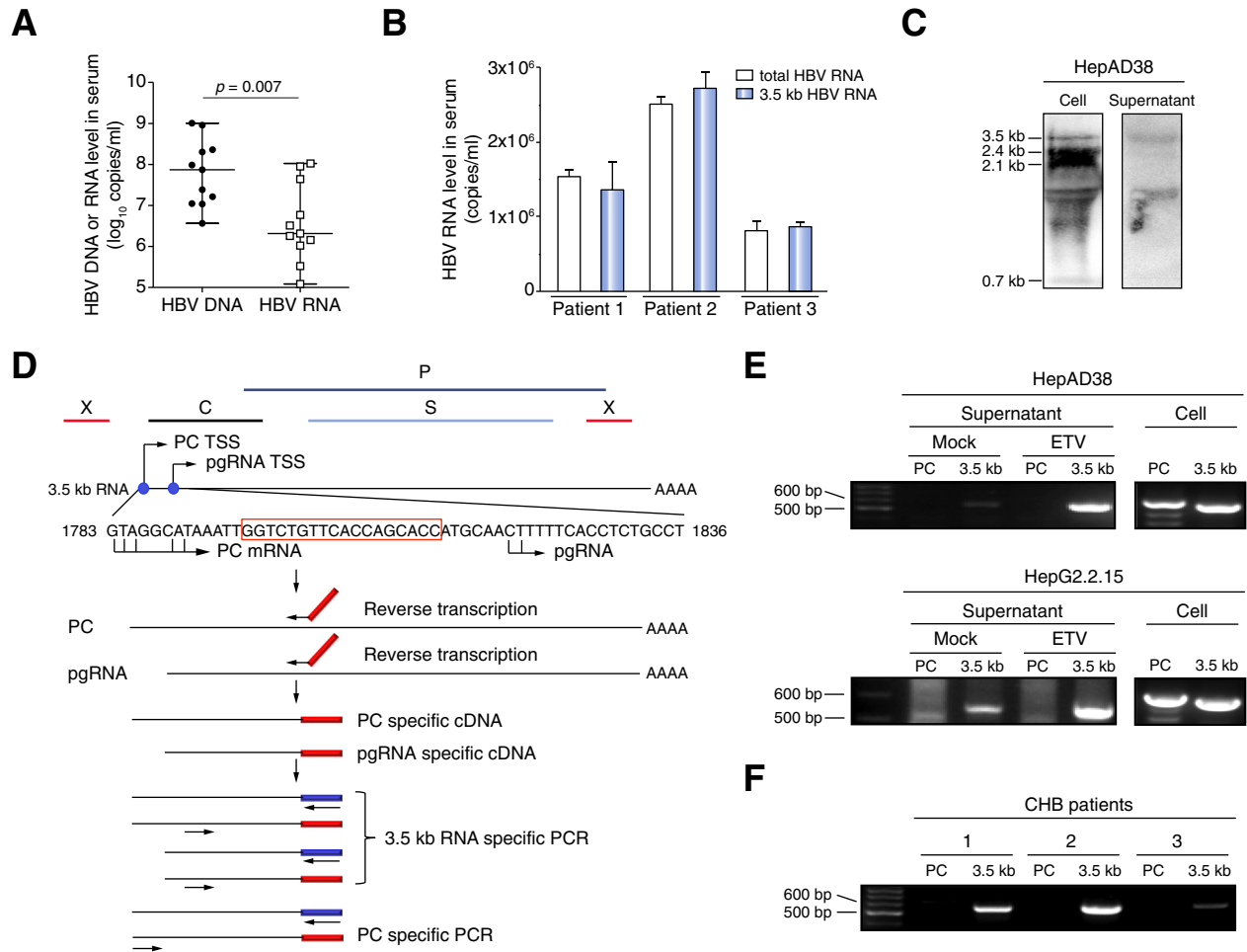


Fig. 1. Identification on the nature of HBV RNA in serum. (A) HBV DNA and HBV RNA levels in the sera of treatment-naïve CHB patients ($n = 11$) were detected by qPCR or RT-qPCR. (B) The levels of total HBV RNA and 3.5 kb HBV RNA in the sera of three treatment-naïve CHB patients were detected by RT-qPCR. (C) HBV RNA in HepAD38 cells or in the supernatant of HepAD38 cells was detected by northern blot. The supernatant (300 ml) of HepAD38 cells was PEG-precipitated to concentrate HBV before extracting HBV RNA. (D) The diagram of multiple identification PCRs, in which the transcription starting sites (TSS) of preC mRNA (PC) and pgRNA (at the nucleotide 1818 or 1819 in HBV genome) are indicated according to the reference sequence of HBV genome from GenBank (Accession AB014381). The sequence of preC mRNA specific forward primer is shown in the red box. (E) The levels of preC mRNA (PC) and 3.5 kb HBV RNA in HepAD38 and HepG2.2.15 cells, the supernatant of HepAD38 and HepG2.2.15 cells, or the sera of three treatment-naïve CHB patients were detected by multiple identification PCRs (F). (This figure appears in colour on the web.)

data provides further supporting evidence that HBV RNA is present in HBV-like viral particles likely as HBV pgRNA virions.

To further confirm that the RNA presented in HBV-like viral particles is pgRNA, the 5' end of HBV RNA in fraction 26 was analyzed using 5'RACE-based PCR and clonal sequencing assay. As shown in Fig. 2G, HBV RNA in fraction 26 was indeed pgRNA transcribed from nucleotide 1818 or 1819 in HBV genome (for details, refer to Fig. 1D).

To provide further evidence that HBV pgRNA virions in supernatants was produced by the encapsidated pgRNA, HepAD38 cells were treated with GLS4, a capsid assembly modifier that interferes with the HBV capsid assembly process and reduces HBV replication [23,29]. Specifically, GLS4 disrupts the HBV lifecycle by inducing the assembly of defective capsids and blocking pgRNA encapsidation, as demonstrated by the decreased level of intracellular encapsidated pgRNA, and consequently intracellular and extracellular encapsidated HBV DNA (Fig. 2H and Supplementary Fig. 4). Extracellular HBeAg level also decreased

due to GLS4-induced inhibition of intracellular encapsidated HBV DNA and subsequently decreased the cccDNA pool (Supplementary Fig. 5). After the treatment of GLS4, the decreased level of pgRNA virions and core protein in supernatant indicated that serum pgRNA virions were packaged by capsid protein (Fig. 2I; Supplementary Fig. 3).

Suppression of the reverse transcription activity of HBV DNA polymerase increases the level of HBV pgRNA virions in vitro

Given that pgRNA is a component of the HBV virion and the only encapsidated HBV RNA in the cytoplasm [30], we hypothesize that HBV pgRNA virion might originate from the non- or partially reverse transcribed pgRNA encapsidated in the cytoplasm. As pgRNA would normally be degraded during the process of reverse transcription, an increase of HBV RNA virion should be expected following inhibition of reverse transcription. We tested this by measuring HBV pgRNA virion levels following NA-mediated block

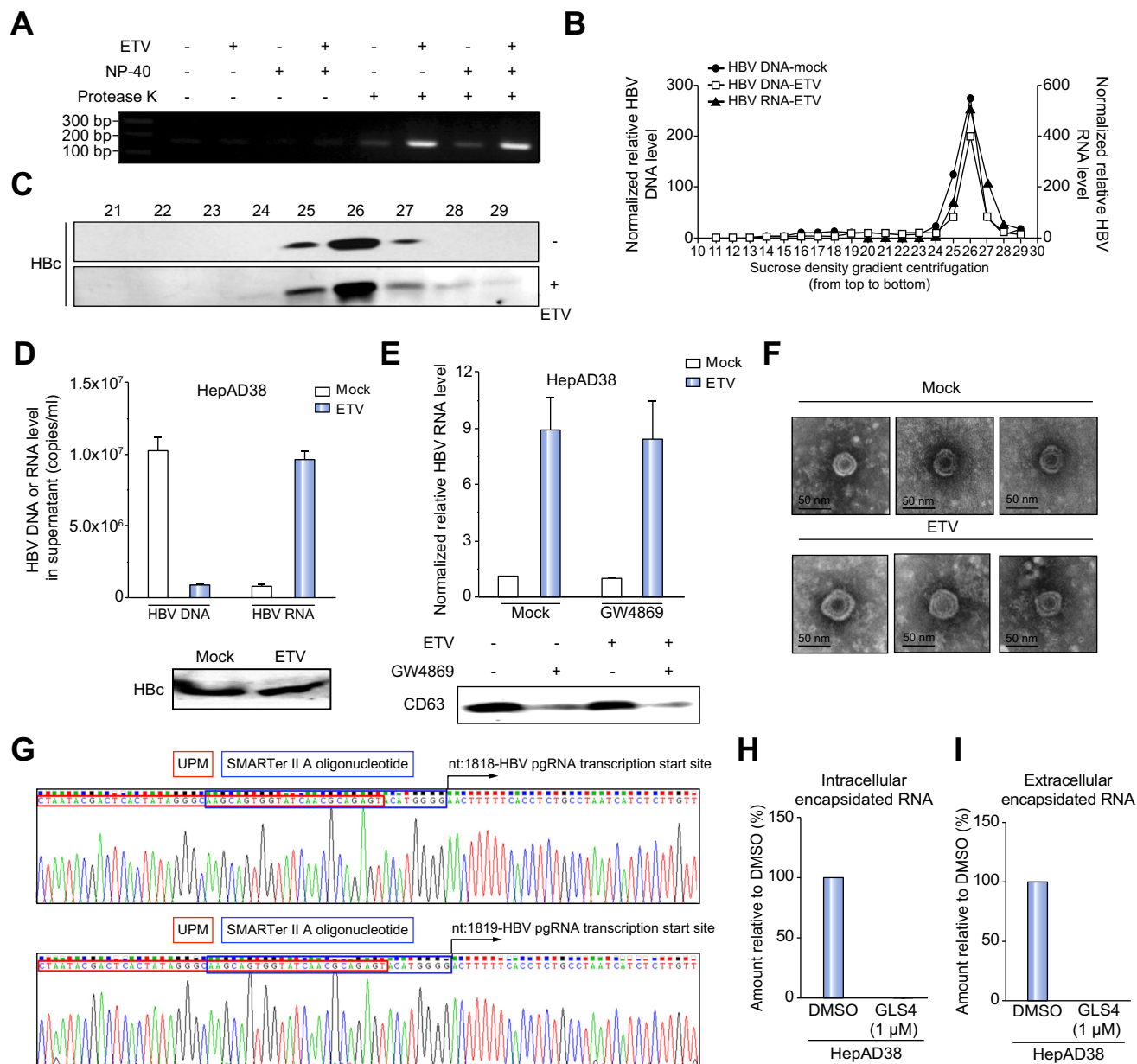


Fig. 2. Analysis on the packaged format of HBV RNA in the supernatant of HepAD38 cells. (A) The 200 μ l supernatants of HepAD38 cells treated with or without ETV were divided into 4 groups, including untreated group 1, group 2 treated with NP-40, group 3 treated with protease K, and group 4 treated with both NP-40 and protease K. Then, HBV RNA was isolated after omitting the step of protease K treatment. The levels of HBV RNA were detected by RT-PCR. (B) To concentrate HBV, the supernatant (100 ml) of mock or ETV-treated HepAD38 cells was performed PEG-precipitation. Then, the levels of HBV DNA and HBV RNA in different fractions prepared by sucrose density gradient centrifugation were detected by qPCR or RT-qPCR (SYBR Green), and the levels of HBcAg (HBc) were detected by Western blot (C). (D) HBV DNA and HBV RNA levels in the supernatant of mock or ETV-treated HepAD38 cells were detected by qPCR and RT-qPCR (TaqMan probe), respectively. After PEG-precipitation, the levels of HBcAg (HBc) in the supernatant of HepAD38 cells were detected by Western blot. The sampling volume was adjusted according to cell number. (E) HBV RNA levels in the supernatant of HepAD38 cells with or without the treatment of GW4869 were detected by RT-qPCR (SYBR Green). CD63 protein levels in the supernatant of HepAD38 cells were detected by Western blot. The sampling volume was adjusted according to cell number. (F) The fraction 26 separated by sucrose density gradient centrifugation for the supernatant of HepAD38 cells with or without the treatment of ETV was observed under electron microscopy. (G) The nature of HBV RNA in fraction 26 was identified by 5'RACE-based PCR and clonal sequencing assay. HepAD38 cells were treated with DMSO or core inhibitor GLS4 (1 μ M) for 6 days. The levels of PEG-precipitated intracellular (H) and supernatant/extracellular (I) pgRNA were detected by RT-qPCR. Statistical analyses were performed by the two groups of related non-parameters Wilcoxon symbols test. (This figure appears in colour on the web.)

in HBV reverse transcription. Indeed, the levels of HBV pgRNA virion in the supernatant increased in a dose-dependent manner when HepAD38 cells were treated with ETV and lamivudine (LMV). A dramatic decrease in the level of HBV DNA was also

observed (Fig. 3A; Supplementary Fig. 6). This effect was also observed in HepG2.2.15 cells (Fig. 3B). The decrease in HBV DNA levels and increase in HBV pgRNA virion levels also occurred in a time-dependent manner in the supernatant of either

Research Article

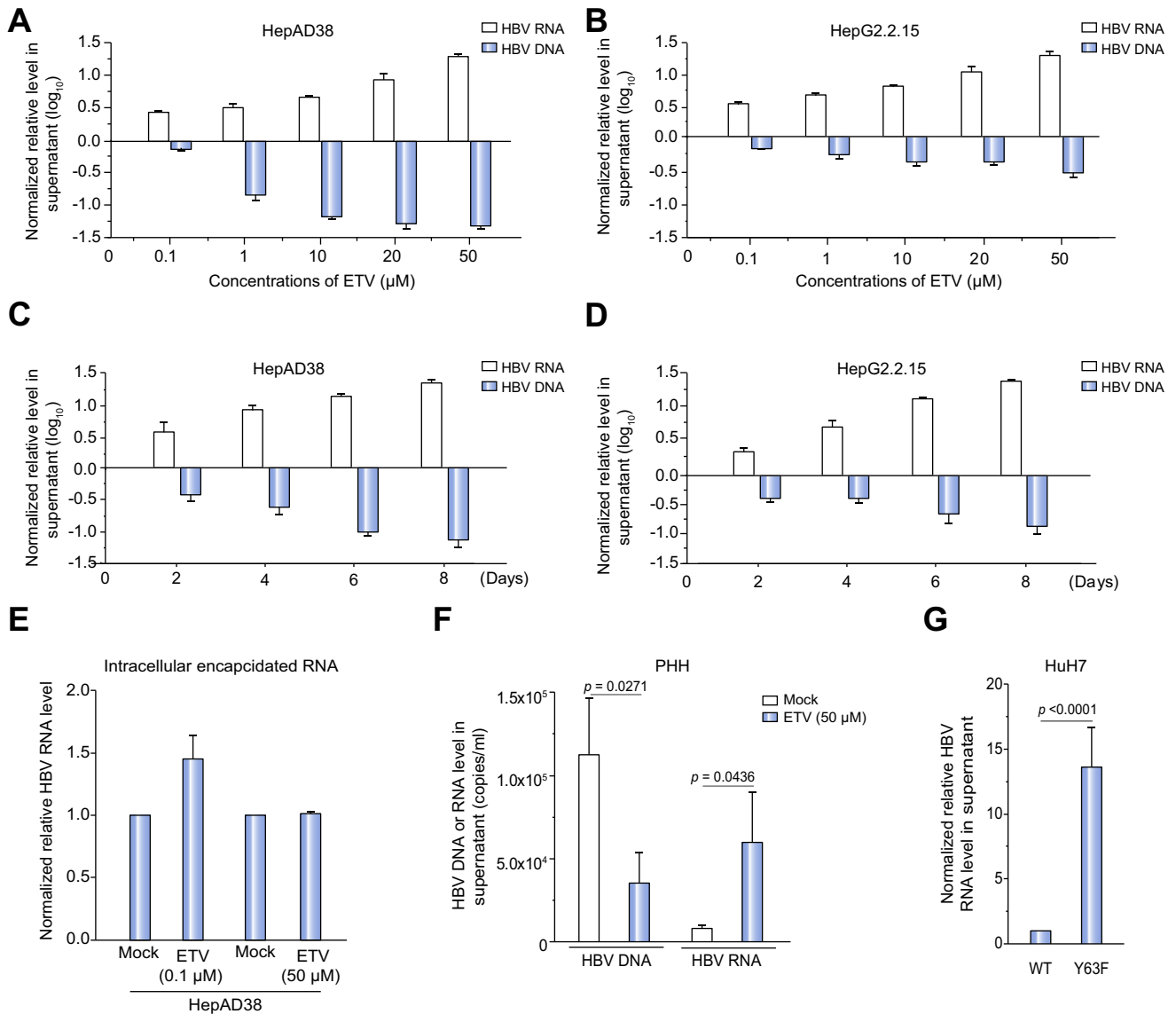


Fig. 3. The origin of HBV pgRNA virion was determined *in vitro*. HBV DNA and HBV pgRNA virion levels in the supernatant of HepAD38 (A) and HepG2.2.15 (B) cells treated with different concentrations (0.1, 1, 10, 20 and 50 μM) of ETV were detected by qPCR and RT-qPCR, respectively. HBV DNA and HBV pgRNA virion levels in the supernatant of HepAD38 (C) and HepG2.2.15 (D) cells treated with ETV (50 μM) for different time (0, 2, 4, 6 and 8 days) were detected by qPCR and RT-qPCR, respectively. (E) The levels of encapsidated HBV RNA in HepAD38 cells treated with ETV (0.1 or 50 μM) for 6 days were detected by RT-qPCR. (F) PHH (1×10^5 cells/well) were seeded in a 48-well plate, and inoculated with 1×10^7 copies of genome equivalent HBV for 20 h in PMM with 4% PEG8000. At 1-day post infection, PHH were treated with ETV for 4 days and HBV pgRNA virion levels in the supernatant were detected by RT-qPCR. (G) HuH7 cells were seeded in a 12-well plate at a density of 4×10^5 cells/ml, and transfected with 1 μg of either wild-type (WT) or Y63F mutant 1.2 × HBV construct. The supernatants of HuH7 cells were harvested at 5 days post transfection, and the levels of HBV pgRNA virion in the supernatant were detected by RT-qPCR.

HepAD38 and HepG2.2.15 cells treated with ETV for different times (2, 4, 6 and 8 days) (Fig. 3C and D). However, cytoplasmic encapsidated HBV RNA levels in HepAD38 cells either remained unchanged or increased upon ETV treatment (Fig. 3E). HBV pgRNA virion levels in the supernatant of primary human hepatocytes (PHH) infected with HBV also significantly increased after ETV treatment (Fig. 3F).

In addition to blocking HBV reverse transcription by NAs, a priming reaction-deficient TP-Y63F mutant (Y63F) was constructed that could encapsidate pgRNA, but could not efficiently

generate rcDNA [31]. Consistent with this concept, the level of HBV pgRNA virion in the supernatant of HuH7 cells transiently transfected with the Y63F mutant construct was significantly higher than that of its wild-type counterpart ($p < 0.001$) (Fig. 3G).

*Suppression of the reverse transcription activity of HBV DNA polymerase increases the levels of HBV pgRNA virion *in vivo**

In addition to the *in vitro* experiments described, the origin of HBV pgRNA virion in HBV transgenic mice was analyzed. Eight

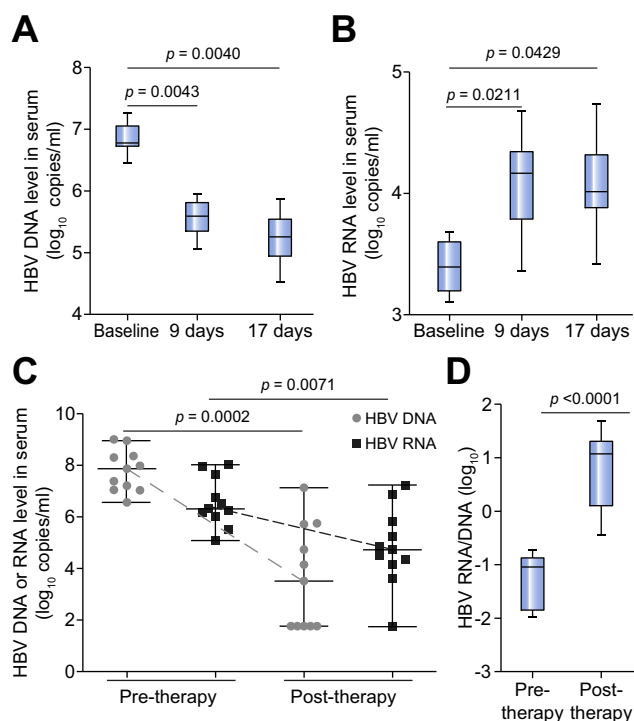


Fig. 4. The origin of HBV pgRNA virion was determined *in vivo*. HBV DNA (A) and HBV pgRNA virion (B) levels in the sera of 6-week-old female HBV transgenic mice ($n = 8$) were detected by qPCR or RT-qPCR at ETV pre-treatment (Baseline), 9 and 17 days post ETV (3.2 mg/kg) treatment. Statistical analyses were performed by the repeated measures analysis of variance. (C) The pre-therapy and post-therapy levels of HBV DNA and HBV pgRNA virions in the sera of CHB patients ($n = 11$) were detected in parallel by qPCR and RT-qPCR, respectively. (D) The pre-therapy and post-therapy HBV RNA/DNA ratios in the sera of CHB patients ($n = 11$). Statistical analyses were performed by the two groups of related non-parameters Wilcoxon symbols test, as the data of the two groups was not subjected to normal distribution and highly relevant.

HBV transgenic mice were treated with 3.2 mg/kg of ETV or a solvent placebo by oral gavage once per day. Blood was sampled from the angular vein at three time points, at ETV pre-treatment (baseline), 9 and 17 days post ETV treatment, and the levels of HBV DNA and HBV pgRNA virion were determined by qPCR and RT-qPCR. As shown in Fig. 4A, the levels of HBV DNA in the sera of HBV transgenic mice significantly decreased at 9 and 17 days post ETV treatment compared to baseline levels ($p = 0.0043$ and 0.0040 , respectively). In contrast, the levels of HBV pgRNA virion increased significantly at the same time points ($p = 0.0211$ and 0.0429 , respectively), consistent with the results of *in vitro* experiments (Figs. 3A and B, 4B). No significant changes of either HBV DNA or HBV pgRNA virion in the sera of the solvent placebo controls were observed (data not shown).

The pre-therapy and post receiving NA-therapy levels of HBV DNA and HBV pgRNA virion in serum were subsequently determined in a cohort of telbivudine (TBV)-treated CHB patients (cohort A, $n = 11$) by qPCR and RT-qPCR, respectively. The level of HBV DNA virion significantly decreased from 7.87 (6.56–9.01) \log_{10} copies/ml at pre-therapy to 3.51 (1.76–7.13) \log_{10} copies/ml ($p = 0.0002$) at different times after receiving NA-therapy. Although the level of HBV pgRNA virion also significantly decreased from 6.31 (5.10–8.02) \log_{10} copies/ml to

4.73 (1.74–7.24) \log_{10} copies/ml after the long-term of TBV treatment ($p = 0.0071$), its level declined much less than HBV DNA (Fig. 4C). Therefore, we use the ratio of HBV pgRNA virion to HBV DNA (HBV RNA/DNA) to reflect the changing pattern, and the ratio was found to be increased significantly following NA-therapy ($p < 0.0001$, Fig. 4D).

Since NA-mediated HBV polymerase inhibition blocks HBV replication through blocking the generation of rcDNA reversely transcribed from pgRNA, they may not inhibit the generation of HBV pgRNA virions. We theorized that the presence of pgRNA virion in patient's serum should reflect the presence and transcriptional activity of cccDNA in patient liver. In order to investigate whether HBV pgRNA virion was associated with risk of viral rebound after the end of treatment (EoT), 33 CHB patients (cohort B) who received standard NAs treatment and with HBV DNA levels below the lower limit of detection (LoD) at EoT were followed up. Among these CHB patients, the levels of serum HBV RNA were positive in 21 (63.64%) patients, and 12 (36.36%) patients had serum HBV RNA levels below the LoD. On the basis of HBV DNA re-appearance at 24 weeks post of EoT, viral rebound occurred in 21 (100%) patients whose HBV RNA was positive at EoT, whereas, only in 3 (25%) of the 12 patients whose HBV RNA levels were below the LoD. It is worthwhile to consider that the level of HBV pgRNA virion might be associated with risk of HBV viral rebound after discontinuation of NA-therapy ($p = 0.001$) (Table 3).

Discussion

This study aimed to thoroughly investigate observations in previous studies suggesting that HBV RNA in the sera of treatment-naïve CHB patients might be pgRNA [9,10]. Because it is generally believed that HBV DNA synthesis is required for nucleocapsid maturation and envelopment [32,33], one may argue that without initiation of reverse transcription, the encapsidated pgRNA/P protein complex could not be enveloped and properly secreted. However, several studies have suggested that the nucleocapsid maturation may not be DNA synthesis-dependent [34–36] and genome-free HBV virions were also detected in the sera of CHB patients, supporting the presence of non-classical HBV packaging pathway in which DNA synthesis may not be needed [37,38].

An important first question to address is the nature of serum HBV RNA detected in CHB patients (Fig. 1). A series of experiments in several cell types and different compounds, revealed the detected serum HBV RNA is only pgRNA. Northern blot identified the RNA as 3.5 kb RNA, then multiple identification PCRs and 5' RACE experiments specifically found the RNA to be pgRNA and not preC mRNA (Figs. 1 and 2). Importantly, this pgRNA originates from encapsidated virions as demonstrated by utilizing proteinase K treatment, sucrose gradient centrifugation, electron microscopy, as well as GLS4 treatment (Fig. 2). Furthermore, we detected HBV pgRNA virions in the supernatants of HepAD38 and HepG2.2.15 cells. Since hepatoma cells have a particular behavior in terms of viral particle secretion, for example, the nonenveloped nucleocapsids are often found after transfection of hepatoma cells with efficient HBV expression constructs [39], we also detected HBV pgRNA virion in supernatants of PHH. It should also be noted that the levels of HBV pgRNA virion increased upon ETV treatment (Fig. 3).

Research Article

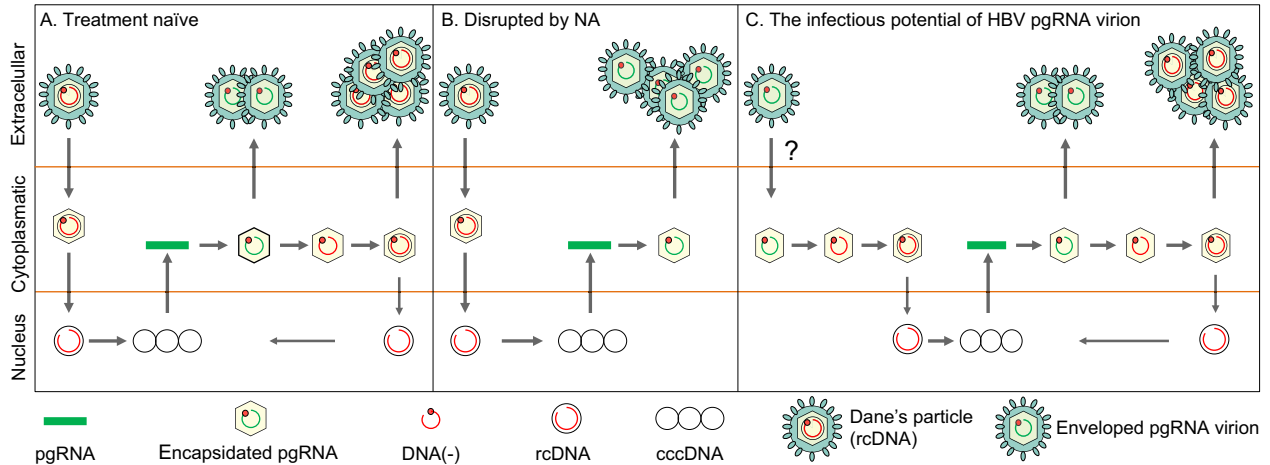


Fig. 5. Model on the production of enveloped pgRNA virion and its infectious potential. (This figure appears in colour on the web.)

The level of HBV pgRNA virion in HBV transgenic mice also significantly increased after ETV treatment (Fig. 4). Importantly, the level of HBV pgRNA virion declined much slower than HBV DNA did in most CHB patients after receiving NA-therapy, and the ratio of HBV pgRNA virions to HBV DNA (HBV RNA/DNA) was significantly increased, indirectly reflecting that the level of pgRNA virion increase. This is further bolstered by the fact that the HBV Y63F mutant, which is deficient for the reverse transcription of pgRNA, but not for its encapsidation, could produce more HBV pgRNA virions than wild-type virions. Collectively, these results indicate that a block in reverse transcription of pgRNA leads to an obvious increase in HBV pgRNA virions extracellularly. Based on these observations, we propose a model where the presence of HBV pgRNA virion provides a supplementary process during the HBV lifecycle (Fig. 5). Encapsidated pgRNA in the cytoplasm of hepatocytes would be enveloped and released into the serum of treatment-naïve CHB patient as HBV pgRNA virion. Following NA-treatment, reverse transcription would be inhibited, and the pgRNA in core particles would still be enveloped and released into the serum, thereby increasing the level of HBV pgRNA virions. Our postulation that HBV pgRNA virions could be both encapsidated and enveloped was further supported by the direct association of HBV pgRNA with HBsAg in the supernatant of HepG2.2.15 cells through immunoprecipitation with HBsAg-specific antibodies. Moreover, the amount of HBV pgRNA precipitated by HBcAg-specific antibodies in the sera of CHB patients increased approximately 100-fold after removal of the HBsAg envelope [9]. These data, together with our current results, suggest that HBV pgRNA virions are both encapsidated and enveloped. Nonetheless, it is important to emphasize that

more evidence is needed to further demonstrate HBV pgRNA virions are indeed enveloped.

Generally, a virus can be classified either as a DNA virus or RNA virus based on the nucleic acid composition of the viral genome. Accordingly, HBV has traditionally been defined as a double-stranded DNA virus. The discovery of HBV pgRNA virion implies that HBV may have another natural virion form in which the nucleic acid is composed of pgRNA, not the rcDNA. It is well known that hepatitis D virus (HDV) is a defective RNA virus that requires HBV envelope proteins for the production of infectious virus particles [40,41]. We hypothesize that similar to HDV, the enveloped HBV pgRNA virion has the potential to infect hepatocytes. Given that the HBV pgRNA virion contains both the P protein and the pgRNA, it is possible that upon entry into hepatocytes HBV rcDNA is reversely transcribed in the cytoplasm from the nucleocapsid pgRNA. Subsequently, core particles containing rcDNA can enter into the nucleus to form cccDNA, or can be enveloped and secreted to form Dane's particle. Thus, it is possible that HBV pgRNA virions can re-infect hepatocytes and contribute to persistence of HBV infection. Unfortunately, at this time, it is technically too difficult to isolate pure HBV pgRNA virions to confirm such a postulation. To further understand the biological relevance of HBV pgRNA virions, critical issues including the phosphorylation status of capsids, overall infectivity and role in immunopathology should be addressed in the future.

A potential clinical significance of HBV pgRNA virions was also observed in this study. Given that NAs have no or little effect on the transcription of cccDNA, it is possible that HBV pgRNA virions may be continuously produced due to the persistence of cccDNA, even if HBV DNA virus is undetectable. Detection of pgRNA virions in serum may reflect the presence and active transcription of cccDNA in the liver of patients, and in the case of NA-therapy, constant undetectable level of serum HBV pgRNA may serve as a predictive biomarker to guide the safe discontinuation of NA-therapy. In this study, serum HBV pgRNA was detected in 33 CHB patients whose HBV DNA virus was undetectable at the EoT. It is important to note that HBV pgRNA virion was still positive in the sera of 21 CHB patients, and viral rebound occurred in all these patients (21/21) at 24 weeks post treatment. Whereas among the 12 CHB patients whose HBV pgRNA virion was

Table 3. The association of HBV pgRNA virion levels and viral rebound after the discontinuation of NAs-therapy.

HBV RNA	Viral rebound (n)	No viral rebound (n)	Total (n)	*p value
Positive	21	0	21	0.001
Below the LoQ	3	9	12	
Total (n)	24	9	33	

*Chi-Square test; n, number of CHB patients.

undetectable, viral rebound only occurred in 3 patients, thereby strongly suggesting that HBV pgRNA virion might be associated with risk of viral rebound after the discontinuation of NA-therapy. It will be an important future task to use a large patient cohort to further determine the association of HBV pgRNA virion with risk of viral rebound.

In conclusion, we performed a series of experiments and identified that HBV RNA in serum is pgRNA that is encapsidated and present in HBV-like viral particles. The discovery of HBV pgRNA virion provides another level of complexity to the HBV lifecycle, where in addition to HBV DNA, encapsidated pgRNA can also be enveloped and secreted out of the cell. Clinically, our data reveals that the level of HBV pgRNA virion in serum may be associated with risk of HBV viral rebound after withdrawal of treatment, and therefore, a potential predictive biomarker to monitor the safe discontinuation of NA-therapy.

Financial support

This work was supported by grants from the Natural Science Foundation of China (No. 81471938), the National S & T Major Project for Infectious Diseases (No. 2013ZX10002-002 and 2012ZX10002-005), and 111 project (B07001). Assembly Biosciences Inc. (ASMB) is a publicly traded US company.

Conflict of interest

Kumar GR, Li PC, Huang Q. and Colonno R. are employees of Assembly Biosciences, Inc., San Francisco, CA, USA. All other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Author's contributions

Lu FM, Wang J, Shen T, Huang XB, Xia NS, Huang Q, Gao ZL and Ren H designed the research; Wang J, Huang XB, Shen T, Chen XM, Zeng ZZ, Zhang RY, Chen R, Li T, Zhang TY, Kumar GR, Li PC and Yuan Q performed the research; all authors analyzed the data; Wang J, Lu FM, Huang XB, Kumar GR, Huang Q and Shen T wrote the paper; Lu FM, McCrae MA, Colonno R, Hou JL, Jia JD and Zhuang H revised the paper.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2016.05.029>.

References

Author names in bold designate shared co-first authorship

- [1] Ott JJ, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 2012;30:2212–2219.

- [2] Mohd HK, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 2013;57:1333–1342.
- [3] Trepo C, Chan HL, Lok A. Hepatitis B virus infection. *Lancet* 2014;384:2053–2063.
- [4] Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. *N Engl J Med* 2004;350:1118–1129.
- [5] Pan CQ, Zhang JX. Natural history and clinical consequences of hepatitis B virus infection. *Int J Med Sci* 2005;2:36–40.
- [6] Schadler S, Hildt E. HBV life cycle: entry and morphogenesis. *Viruses* 2009;1:185–209.
- [7] Kock J, Theilmann L, Galle P, Schlicht HJ. Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus. *Hepatology* 1996;23:405–413.
- [8] Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. *Clin Cancer Res* 2001;7:2005–2015.
- [9] Jansen L, Kootstra NA, van Dort KA, Takkenberg RB, Reesink HW, Zaaier HL. Hepatitis B virus pregenomic RNA is present in virions in plasma and is associated with a response to pegylated interferon alfa-2a and nucleos(t)ide analogues. *J Infect Dis* 2016;213:224–232.
- [10] Tsuge M, Murakami E, Imamura M, Abe H, Miki D, Hiraga N, et al. Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients. *J Gastroenterol* 2013;48:1188–1204.
- [11] Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, et al. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 2007;45:1179–1186.
- [12] Rokuhara A, Matsumoto A, Tanaka E, Umemura T, Yoshizawa K, Kimura T, et al. Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. *J Gastroenterol* 2006;41:785–790.
- [13] Huang YW, Takahashi S, Tsuge M, Chen CL, Wang TC, Abe H, et al. On-treatment low serum HBV RNA level predicts initial virological response in chronic hepatitis B patients receiving nucleoside analogue therapy. *Antivir Ther* 2015;20:369–375.
- [14] van Bommel F, Bartens A, Mysickova A, Hofmann J, Kruger DH, Berg T, et al. Serum hepatitis B virus RNA levels as an early predictor of hepatitis B envelope antigen seroconversion during treatment with polymerase inhibitors. *Hepatology* 2015;61:66–76.
- [15] **Lucifora J, Xia Y**, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 2014;343:1221–1228.
- [16] Wang J, Xu ZW, Liu S, Zhang RY, Ding SL, Xie XM, et al. Dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication. *World J Gastroenterol* 2015;21:9554–9565.
- [17] Zoulim F. Hepatitis B virus resistance to antiviral drugs: where are we going? *Liver Int* 2011;31:111–116.
- [18] Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res* 1982;42:3858–3863.
- [19] Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci U S A* 1987;84:1005–1009.
- [20] Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, 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;41:1715–1720.
- [21] **Yan H, Zhong GC**, Xu GW, He WH, Jing ZY, Gao ZC, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* 2012;1 e00049.
- [22] **Wu MH, Ma WL, Hsu CL**, Chen YL, Ou JH, Ryan CK, et al. Androgen receptor promotes hepatitis B virus-induced hepatocarcinogenesis through modulation of hepatitis B virus RNA transcription. *Sci Transl Med* 2010;2:32ra35.
- [23] Wang XY, Wei ZM, Wu GY, Wang JH, Zhang YJ, Li J, et al. In vitro inhibition of HBV replication by a novel compound, GLS4, and its efficacy against adefovir-dipivoxil-resistant HBV mutations. *Antivir Ther* 2012;17:793–803.
- [24] Chen RY, Edwards R, Shaw T, Colledge D, Delaney WT, Isom H, et al. Effect of the G1896A precore mutation on drug sensitivity and replication yield of lamivudine-resistant HBV in vitro. *Hepatology* 2003;37:27–35.
- [25] Gripon P, Diot C, Guguen-Guillouzo C. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology* 1993;192:534–540.

Research Article

- [26] Glebe D, Gerlich WH. Study of the endocytosis and intracellular localization of subviral particles of hepatitis B virus in primary hepatocytes. *Methods Mol Med* 2004;96:143–151.
- [27] **Bukong TN, Momen-Heravi F**, Kodys K, Bala S, Szabo G. Exosomes from hepatitis C infected patients transmit HCV infection and contain replication competent viral RNA in complex with Ago2-miR122-HSP90. *PLoS Pathog* 2014;10 e1004424.
- [28] Chahar HS, Bao X, Casola A. Exosomes and their role in the life cycle and pathogenesis of RNA viruses. *Viruses* 2015;7:3204–3225.
- [29] Wu G, Liu B, Zhang Y, Li J, Arzumanyan A, Clayton MM, et al. Preclinical characterization of GLS4, an inhibitor of hepatitis B virus core particle assembly. *Antimicrob Agents Chemother*. 2013;57:5344–5354.
- [30] Hu J, Seeger C. Hepadnavirus genome replication and persistence. *Cold Spring Harb Perspect Med* 2015;5:a21386.
- [31] Wang JC, Nickens DG, Lentz TB, Loeb DD, Zlotnick A. Encapsidated hepatitis B virus reverse transcriptase is poised on an ordered RNA lattice. *Proc Natl Acad Sci U S A* 2014;111:11329–11334.
- [32] Gerelsaikhan T, Tavis JE, Bruss V. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J Virol* 1996;70:4269–4274.
- [33] Roseman AM, Berriman JA, Wynne SA, Butler PJ, Crowther RA. A structural model for maturation of the hepatitis B virus core. *Proc Natl Acad Sci U S A* 2005;102:15821–15826.
- [34] Gerin JL, Ford EC, Purcell RH. Biochemical characterization of Australia antigen. Evidence for defective particles of hepatitis B virus. *Am J Pathol* 1975;81:651–668.
- [35] Kaplan PM, Ford EC, Purcell RH, Gerin JL. Demonstration of subpopulations of Dane particles. *J Virol* 1976;17:885–893.
- [36] Sakamoto Y, Yamada G, Mizuno M, Nishihara T, Kinoyama S, Kobayashi T, et al. Full and empty particles of hepatitis B virus in hepatocytes from patients with HBsAg-positive chronic active hepatitis. *Lab Invest* 1983;48:678–682.
- [37] Ning X, Nguyen D, Mentzer L, Adams C, Lee H, Ashley R, et al. Secretion of genome-free hepatitis B virus-single strand blocking model for virion morphogenesis of para-retrovirus. *PLoS Pathog* 2011;7 e1002255.
- [38] Luckenbaugh L, Kitrinos KM, Delaney WT, Hu J. Genome-free hepatitis B virion levels in patient sera as a potential marker to monitor response to antiviral therapy. *J Viral Hepat* 2015;22:561–570.
- [39] Ren S, Nassal M. Hepatitis B virus (HBV) virion and covalently closed circular DNA formation in primary tupaia hepatocytes and human hepatoma cell lines upon HBV genome transduction with replication-defective adenovirus vectors. *J Virol* 2001;75:1104–1116.
- [40] Littlejohn M, Locarnini S, Yuen L. Origins and evolution of hepatitis B virus and hepatitis D virus. *Cold Spring Harb Perspect Med* 2016;6:a021360.
- [41] Huang CR, Lo SJ. Hepatitis D virus infection, replication and cross-talk with the hepatitis B virus. *World J Gastroenterol* 2014;20:14589–14597.