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

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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 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.

Keywords: Hepatitis B virus; HBV pgRNA virion; Nucleot(s)ide analogues therapy.

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.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>7:4</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39 (24-52)</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>141 (85-318)</td>
</tr>
<tr>
<td>HBV DNA (log_{10} copies/ml)</td>
<td>7.87 (6.56-9.01)</td>
</tr>
<tr>
<td>HBsAg (IU/ml)</td>
<td>15,695 (1221-196,392)</td>
</tr>
<tr>
<td>HBeAg (S/CO)</td>
<td>1846 (83-4528)</td>
</tr>
<tr>
<td>NAs</td>
<td>LdT</td>
</tr>
<tr>
<td>Duration of NAs therapy (weeks)</td>
<td>236 (24-268)</td>
</tr>
</tbody>
</table>

*Median (range). NAs, nucleot(s)ide analogues; TBV, telbivudine.

### Table 2. Clinical background of the patient cohort B.

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

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.
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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, G196RA 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'-CTGGCAAAATCTTACCCGTCTTCTCTCTGAC-3' and probe: 5'-TACCTTTAATCC-3'. Reverse: 5'-GGATTAAAGATACAGTAGAAGAGAAAAGCCCAGTA AAGTTTCCCAC-3.'

HBV infection

HBV infection of PHI was conducted as previously described (25). Briefly, 1 x 10^5 PHH cells in a 48-well plate were inoculated with 1 x 10^5 copies of genome equivalent HBV in the presence of 4% PEG 8000 for 2 h. PHH cells were then washed with PBS six times and maintained in PPM 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 ReverTraActM First Strand DNA Synthesis Kit (Toho Bio-Pharm, Tokyo, Japan) with either random primers or an HBV specific RT primer. The sequence of HBV specific RT primer was: 5'-ATTCTCACAGCTTAGCACAGCACCCAGACACCTTACCCACGAC-3' in which the random sequence ATTCTCACAGCTTAGCACACGACAC was anchored at the 5' end of HBV specific sequence GGAGATGAGATCTTTCGCCGAC (nucleotide [nt] 2436-2415) and used for detection of the 3.5 kb HBV RNA, or: 5'-ATTCTCACAGCTTAGCACAGCACCCAGACACCTTACCCACGAC-3' in which the random sequence ATTCTCACAGCTTAGCACACGACAC was anchored at the 3' end of HBV specific sequence GGCCTCAAGGTCGGTCGTTGAC (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 preC RNA, 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'-CTTACGTCAACGCCACACGAC-3' and F (nt 1796-1813): 5'-GGCTTGTCACACACACCCG-3', respectively. Both of the reverse primer was the anchored random sequence: 5'-ATTCTTAGCCACTGACACGACAC-3'. PCR reaction mixture (20 μl) contained 10 μl 2 x Taq mix (GenStar, Beijing, China), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl cDNA template and 7 μl double distilled water (ddH2O). The reaction mixture was denatured at 95°C for 5 min followed by 35 cycles at 95°C for 20 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 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'-ATTCTCAGACCGTAGCACACGACAC-3' and probe: 5'-ATTCTCAGACCGTAGCACACGACAC-3'. PCR reaction mixture (30 μl) contained 15 μl 2 x mix (LightCycler®480 Probes Master, Roche, Mannheim, Germany), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl TaqMan probe (10 μM), 3 μl cDNA template and 9 μl 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 45 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 fluorimunoassay (TRFIA) as previously described (16) or by a commercial enzymelinked immunosorbsent 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 H1091 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-TECNAL12, New York, USA) at 80 kV.

Northern blot

TRZol® 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 in 2 x SSC/0.1% sodium dodecyl sulfate, and then 0.5 x saline sodium citrate/0.1xSSS 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 2% 2-mercaptoethanol), boiled for 10 min at 95°C and resolved on 4–20% gradient SDS-PAGE gel (BioRad). HBV core protein was resolved on 4–20% gradient SDS-PAGE gel (BioRad). HBV core protein was transferred to nitrocellulose membrane and blocked in 5% non-fat milk in PBS-Tween 20 for 1 h. The membrane was incubated with primary anti-HBV Core antibody (polyclonal, provided by Dr. Locarnini) for 1 h, followed by rinsing with PBS-Tween 20 for 1 min and incubating in 1:5000 diluted HRP- conjugated secondary antibody in PBS-Tween 20 for 1 h. Blots were washed with PBS-Tween 20 for 3 min, and then exposed to X-ray film (Kodak X-OMAT3).
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-qPCR (RT-PCR). 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 protease K to destroy the proteinaceous structure. HBV RNA was only detected in the supernatant treated with protease K, indicating that HBV RNA in HepAD38 supernatant was contained within a proteinaceous structure. HBV RNA was only detected in the supernatant treated with protease 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 protease 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, HBcAg 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, HBcAg 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 HBcAg, 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 HBcAg levels in the supernatant of ETV-treated HepAD38 cells. We postulated that if HBV particles consist of only HBV DNA, then the levels of HBcAg should decline to the same degree as that of HBV DNA in ETV-treated HepAD38 cell supernatants. Results show that HBcAg 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 ± 0.03 log_{10} copies/ml) compared to untreated cells (7.01 ± 0.04 log_{10} copies/ml), the levels of HBV RNA increased more than 10-fold (5.91 ± 0.06 log_{10} copies/ml vs. 7.00 ± 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 ± 0.03 log_{10} copies/ml) was almost equal to that of HBV DNA levels in untreated HepAD38 cell supernatants (7.01 ± 0.04 log_{10} copies/ml). These results combined with the observation that HBcAg 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
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 were 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...
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

Fig. 2. Analysis on the packaged format of HBV RNA in the supernatant of HepAD38 cells. (A) The 200 μl 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 μl) of mock or ETV-treated HepAD38 cells was performed for sucrose density gradient centrifugation (from top to bottom). 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. 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 μM) 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-paramaters Wilcoxon symbols test. (This figure appears in colour on the web.)
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
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 (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.

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).
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 HBsAg-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

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

<table>
<thead>
<tr>
<th>HBV RNA</th>
<th>Viral rebound (n)</th>
<th>No viral rebound (n)</th>
<th>Total (n)</th>
<th>*p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>21</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Below the LoQ</td>
<td>3</td>
<td>9</td>
<td>12</td>
<td>0.001</td>
</tr>
<tr>
<td>Total (n)</td>
<td>24</td>
<td>9</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

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

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

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Conflict of interest
Kumar GR, Li PC, Huang Q, and Colombo 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 YG, 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, Colombo R, Hou JL, Jia JD and Zhuang H revised the paper.

Supplementary data
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References
Author names in bold designate shared co-first authorship


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