HEPATOLOGY

HEPATOLOGY, VOL. 71, NO. 2, 2020

Hepatitis B Virus Virions Produced Under Nucleos(t)ide Analogue Treatment Are Mainly Not Infectious Because of Irreversible DNA Chain Termination

Yongzhen Liu,[1*] Hui Liu,[1*] Zhanying Hu,[2*] Yang Ding,[3*] Xiao-Ben Pan,[4] Jun Zou,[1] Jingyuan Xi,[1] Guangxin Yu,[1] Hongxin Huang,[1] Meng-Ting Luo,[5] Fang Guo,[6] Shuang Liu,[7] Qiuju Sheng,[3] Jidong Jia,[8] Yong-Tang Zheng,[5] Jie Wang,[1] Xiangmei Chen,[1] Ju-Tao Guo,[2] Lai Wei,[9] and Fengmin Lu[1,10]

Nucleos(t)ide analogues (NAs) have been widely used for the treatment of chronic hepatitis B (CHB). Because viral DNA polymerase lacks proofreading function (3′ exonuclease activity), theoretically, the incorporated NAs would irreversibly terminate viral DNA synthesis. This study explored the natures of nascent hepatitis B virus (HBV) DNA and infectivity of progeny virions produced under NA treatment. HBV infectivity was determined by infection of HepG2-NTCP cells and primary human hepatocytes (PHHs). Biochemical properties of HBV DNA in the progeny virions were investigated by qPCR, northern blotting, or Southern blotting hybridization, sucrose gradient centrifugation, and *in vitro* endogenous DNA polymerase assay. Progeny HBV virions produced under NA treatment were mainly not infectious to HepG2-NTCP cells or PHHs. Biochemical analysis revealed that under NA treatment, HBV DNA in nucleaocapsids or virions were predominantly short minus-strand DNA with irreversible termination. This finding was supported by the observation of first disappearance of relaxed circular DNA and then the proportional decline of HBV-DNA levels corresponding to the regions of PreC/C, S, and X genes in serial sera of patients receiving NA treatment. *Conclusion:* HBV virions produced under NA treatment are predominantly replication deficient because the viral genomes are truncated and elongation of DNA chains is irreversibly terminated. Clinically, our results suggest that the viral loads of CHB patients under NA therapy vary with the different regions of genome being detected by qPCR assays. Our findings also imply that NA prevention of perinatal and sexual HBV transmission as well as infection of transplanted livers works not only by reducing viral loads, but also by producing noninfectious virions. (Hepatology 2020;71:463-476).

Hepatitis B virus (HBV) is a major causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[1,2] With an estimated 257 million chronic carriers worldwide,[3] HBV infection remains to be one of the major health problems. HBV belongs to the *Hepadnavividae* family and contains different forms of genome in virions. The Dane particles contain a partial double-strand relaxed circular (rc) DNA of ~3.2 kilobases in length,[4] while a small fraction of virions contain

Abbreviations: ADV, adefovir; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; dNTP, deoxynucleoside triphosphate; Dox, doxycycline; DR, direct repeat; ETV, entecavir; FBS, fetal bovine serum; HBc, HBV core; HBeAg, hepatitis B e antigen; HBIG, hepatitis B immune globulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IC_{50}, half maximal inhibitory concentration; IFN, interferon; LdT, telbivudine; MOI, multiplicity of infection; NA, nucleos(t)ide analogue; nt, nucleotide(s); NTCP, sodium taurocholate cotransporting polypeptide; P protein, polymerase protein; PEG8000, polyethylene glycol 8000; Peg-IFN, pegylated interferon; PFA, phosphonoformic acid; PHHs, primary human hepatocytes; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA; TDF, tenofovir disoproxil fumarate; α-^{32}P-UTP, uridine 5′-[alpha-32P] triphosphate tetra(triethylammonium) salt.

Received January 26, 2019; accepted July 1, 2019.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30844/suppinfo.

Supported by grants from the National S & T Major Project for Infectious Diseases of China (Nos. 2017ZX10302201, 2017ZX10201201, 2017ZX10202203 and 2017ZX10202202), the Natural Science Foundation of China (Nos. 81672013, 81572366), and Beijing Natural Science Foundation (7182080).

**These authors contributed equally to the presented work.*

pregenomic (pg) RNA or the reverse-transcription intermediates, that is, a 3′ truncated pgRNA and a short minus-strand DNA.[5,6]

During HBV infection, the virion enters hepatocyte by binding to its specific receptor, sodium taurocholate cotransporting polypeptide (NTCP).[7] Then, the rcDNA was converted to covalently closed circular DNA (cccDNA) in the nuclei of hepatocyte to serve as the template for transcription of viral RNAs, including pgRNA, which can be used as either mRNA for viral core protein and polymerase protein (P protein) or the template of reverse-transcriptional replication of viral DNA.[8,9] In the cytoplasm, binding of P protein to a stem-loop (epsilon) structure near the 5′ end of pgRNA initiates nucleocapsid assembly and prime minus-strand DNA synthesis at the bulge region of epsilon structure.[10] The priming reaction terminates following the synthesis of only three to four nucleotides (nt), which are subsequently transferred to a complementary sequence motif near the 3′ terminus of pgRNA (first switch) to continue the elongation of minus-strand DNA.[11] During the elongation of minus-strand DNA, pgRNA template is concomitantly degraded by the RNase H domain of viral P protein.[12,13] Upon completion of minus-strand DNA synthesis, the 5′-capped 18 nt of pgRNA escaped from RNase H digestion is transferred from the 3′ terminus of newly synthesized minus-strand DNA to direct repeat (DR) 2 region (second switch)

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.30844

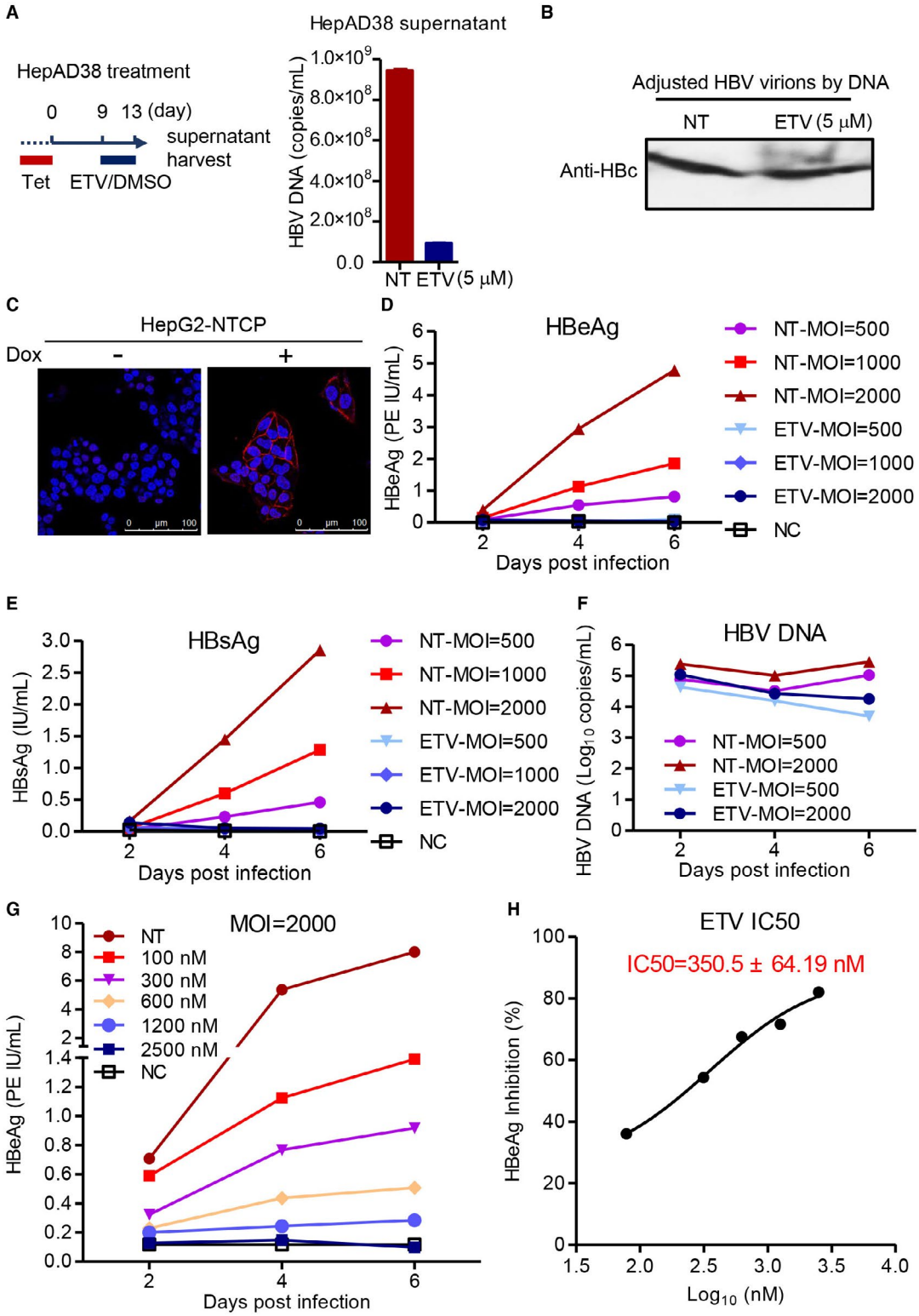
Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the [1]State Key Laboratory of Natural and Biomimetic Drugs, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, P.R. China; [2]Baruch S. Blumberg Institute, Doylestown, PA; [3]Department of Infectious Diseases, Shengjing Hospital of China Medical University, Shenyang, P.R. China; [4]Hangzhou Key Laboratory of Inflammation and Immunoregulation, Department of Basic Medical Science, School of Medicine, Hangzhou Normal University, Hangzhou, P.R. China; [5]Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China; [6]China Novartis Institutes for BioMedical Research, Zhangjiang Hi-Tech Park, Shanghai, P.R. China; [7]Beijing Artificial Liver Treatment & Training Center, Beijing Youan Hospital, Capital Medical University, Beijing, P.R. China; [8]Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing, P.R. China; [9]Hepatopancreatobiliary Center Beijing Tsinghua Changgung Hospital, Tsinghua University, Beijing, P.R. China; [10]Academy of Medical Science, Zhengzhou University, Zhengzhou, P.R. China.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Fengmin Lu, M.D.
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, P.R. China
E-mail: lu.fengmin@hsc.pku.edu.cn
Tel.: +86-10-82805136
or
Lai Wei, M.D.
Hepatopancreatobiliary Center
Beijing Tsinghua Changgung Hospital
Tsinghua University
No. 168 Litang Road
Beijing 102218, P.R. China
E-mail: weilai@mail.tsinghua.edu.cn
Tel.: +86-10-56118923
or
Ju-Tao Guo, M.D.
Baruch S. Blumberg Institute
3805 Old Easton Road
Doylestown, PA, 18902
E-mail: ju-tao.guo@bblumberg.org
Tel.: +1-215-489-4929
or
Xiangmei Chen, Ph.D.
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, P.R. China
E-mail: xm_chen6176@bjmu.edu.cn
Tel.: +86-01-8280-2807



and primes the synthesis of plus-strand DNA.[14] To form circularized rcDNA, the P protein switches template from the 5′ end to the 3′ end of minus-strand DNA (third switch) and continues plus-strand DNA chain elongation. The rcDNA-containing nucleocapsids can either be enveloped and exported as progeny virions

FIG. 1. HBV virions produced under NA treatment were mainly disabled to infect HepG2-NTCP cells. (A) Schematic for the treatment of HepAD38 cells and HBV-DNA quantification of the supernatant with or without ETV treatment. (B) HBV capsid level with adjusted HBV DNA was detected by western blotting of HBV core (HBc) protein. (C) Immunofluorescence demonstration of NTCP expression in HepG2-NTCP cells cultured in the presence of Dox (right). (D-F) HBeAg, HBsAg, and HBV DNA in the media of HepG2-NTCP cultures infected with virions produced by HepAD38 cells treated with DMSO (NT) or ETV at the indicated MOI were tested. Negative control (NC) is media harvested from uninfected cells. (G) HBeAg levels in the media of HepG2-NTCP cultures infected with virions produced by HepAD38 cells treated with different concentrations (0, 100, 300, 600, 1,200, and 2,500 nM) of ETV were detected 2, 4, and 6 days postinfection, respectively. (H) The IC_{50} of ETV in inhibiting progeny HBV infectivity was calculated by nonlinear regression of log (inhibitor) versus response. PE IU/mL, Paul Ehrlich Institute Units.

or recycled back to the nuclei of hepatocytes to replenish the cccDNA pool.[9] The failure of second switch results in *in situ* priming of plus-strand DNA synthesis and production of double-strand linear DNA, which is the preferred precursor for integrated HBV DNA in host cellular chromosomes.[15]

Current therapeutics for chronic hepatitis B (CHB) include immunomodulatory agents (interferon [IFN]-based therapies) and orally available nucleos(t)ide analogues (NAs) that specifically inhibit HBV-DNA polymerase.[16] Unfortunately, neither IFN-α nor NAs can eradicate HBV and cure CHB. Considering the excellent safety and tolerability, NA therapy is often preferred. The currently available NAs include lamivudine, entecavir (ETV), adefovir (ADV), and two prodrugs of tenofovir: tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide.[17-21] Biochemically, those triphosphated NAs compete with endogenous deoxynucleoside triphosphates (dNTPs) for incorporation into viral DNA chain and terminate its elongation immediately,[22] or at 2 or 3 nt downstream of incorporation (ETV).[23] Because viral DNA polymerase lacks proofreading function (3′ exonuclease activity), the incorporated NAs cannot be removed and thus irreversibly terminate viral DNA synthesis. Such prematurely terminated viral DNA would result in the generation of incomplete dead-end HBV DNA in intracellular nucleocapsids and secreted virion-like particles. We noticed, in a recent work that, in spite of approximately 10^4 copies/mL of HBV-DNA–containing virions detected in serum specimens of pregnant women at the time of delivery, NA treatment could successfully eradicated the mother-to-infant vertical transmission.[24] This phenomenon impelled us to investigate whether HBV virions produced under NA therapy are infectious. Our studies suggested that HBV DNA produced in the presence of NA treatment were predominantly 3′ truncated minus-strand DNA with irreversibly incorporated NAs to block its elongation and therefore were mainly not infectious.

Materials and Methods

CELL CULTURE

The HepAD38 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 400 μg/mL of G418.[25] When HepAD38 cells were used for HBV collection, tetracycline was removed from the cell culture for 9 days and then treated with ETV (Fujian Cosunter pharmaceutical company, Fuzhou, China) or dimethyl sulfoxide (DMSO) for 4 days. HepG2-NTCP cells[24] were maintained in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 1 μg/mL of puromycin. Expression of NTCP in this cell line is tetracycline inducible. PHHs were obtained from the BioreclamationIVT (BioIVT, Westbury, NY) and cultured first in PHH Recovery Medium and then were maintained in PHH Maintenance Medium (Liver Biotechnology, Shenzhen, China).

PATIENT SPECIMENS

Individuals for study presented in Fig. 2C,D included 3 CHB patients and 1 healthy serum donor who did not have hepatitis B surface antibody (HBsAb) or any markers of HBV infection in Shengjing Hospital affiliated to China Medical University. Two patients were treatment naïve while the third patient had received TDF therapy for 4 weeks. Clinical information is shown in Supporting Table S1. Patients for study presented in Fig. 3E,F included 10 pregnant women who had received telbivudine (LdT) therapy for 12 weeks until delivery in Shengjing Hospital affiliated to China Medical University. The clinical information is shown in Table 1. Patients for study presented in Fig. 5 included 18 CHB patients who had received

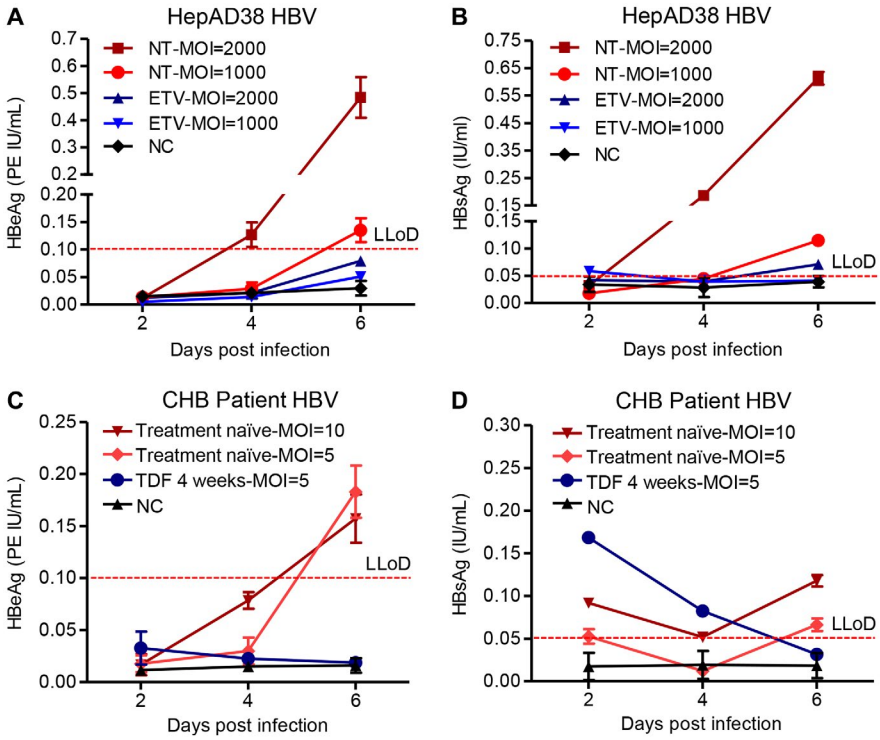


FIG. 2. The virions produced under NA treatment were mainly disabled to infect PHHs. (A,B) HBeAg and HBsAg levels in the media of PHH cultures infected with virions produced by HepAD38 cells treated with DMSO (NT) or 2.5 μM of ETV were detected 2, 4, and 6 days postinfection, respectively. (C,D) HBeAg and HBsAg levels in the media of PHH cultures infected with virions from sera of CHB patients with or without NA therapy were detected 2, 4, and 6 days postinfection, respectively. Negative control (NC) is media harvested from uninfected cells. The dashed lines indicated the LLoD of HBeAg and HBsAg, respectively. PE IU/mL, Paul Ehrlich Institute Units.

ADV therapy for 48 weeks in Peking University People's Hospital, which has been described.[26] Patients for the study presented in Supporting Fig. S6 included 24 CHB patients who had received pegylated (Peg-IFN) IFN-α-2a/b therapy for 48 weeks, and the clinical information is shown in Supporting Table S2. This study was approved by the Ethics Committee of Peking University Health Science Center, as well as the Ethics Committee of People's Hospital, Peking University. Written informed consent was obtained from each patient.

POLYETHYLENE GLYCOL PRECIPITATION

The 6× polyethylene glycol 8000 (PEG8000) buffer (48% PEG8000 and 200 mM of NaCl) was gently added into the culture medium of HepAD38 cells at a volume ratio of 1 to 5. The mixture was shaken slowly overnight at 4°C and then centrifuged at 7,000*g* with the brake "5" for 30 minutes at 10°C in an Eppendorf HL019 rotor (Centrifuge 5810 R; Eppendorf, Hamburg, Germany). The pellet was dissolved with DMEM and proceeded for infection of HepG2-NTCP cells.

HBV INFECTION

HBV infection of HepG2-NTCP cells was conducted as described.[27] Briefly, cells were seeded into collagen-I–coated plates and maintained in DMEM medium containing 4 μg/mL of doxycycline (Merck, Kenilworth, NJ) for 3 days. Cells were then cultured with hepatocyte culture medium (Lonza, Walkersville, MD) for 24 hours and followed by infection with HBV at the indicated multiplicity of infection (MOI) in the presence of 4% PEG8000 for 24 hours. Infected cultures were washed with

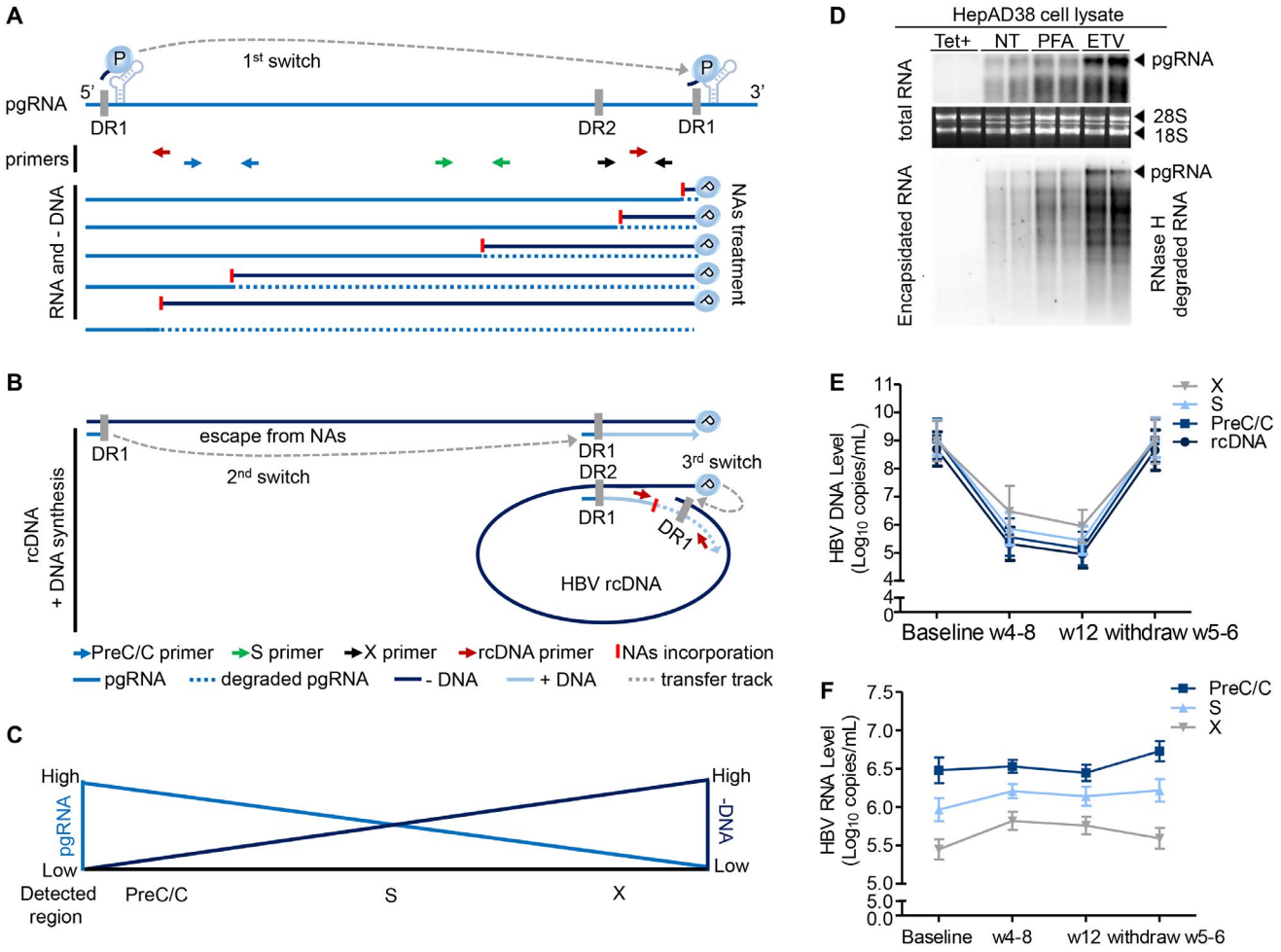


FIG. 3. Effects of NAs on HBV-RNA metabolism and DNA synthesis. (A) Schematic presentation of the effects of NAs on reverse transcription. Under NA treatment, termination of *de novo* HBV-DNA synthesis results in variable length of 3′ truncated minus-strand DNA. Meanwhile, the template pgRNA is degraded by the RNase H activity of P protein. (B) Plus-strand DNA synthesis, genome circularization, and rcDNA synthesis are illustrated. (C) Predicted abundance of HBV DNA and RNA determined by using indicated pairs of PCR primers under NA treatment. (D) Northern blotting analysis of total and encapsidated HBV RNA extracted from HepAD38 cells maintained in the culture without tetracycline but with mock-treated (NT) or treated with 2 mM of PFA or 0.1 μM of ETV. The group with HepAD38 cells maintained in the culture of 0.1 μg/mL of tetracycline (tet+) was set as a negative control. Northern blotting hybridization was performed with an α-^{32}P-UTP–labeled full-length minus-strand HBV RNA. Ribosomal RNA served as loading controls. (E,F) HBV DNA and RNA in the serum of pregnant women under LdT treatment were determined by qPCR assays using the primers specifically amplifying the X, S, and PreC/C regions and rcDNA.

phosphate-buffered saline (PBS) four times and maintained in fresh DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 4 μg/mL of doxycycline, and 2% DMSO with medium change at every other day. For infection of PHHs, PHHs were seeded into collagen-I–coated 24-well plates at a density of 1.5 × 10^5 cells/well. These cultured PHHs were infected with HBV virions collected either from HepAD38 cell-culture supernatant or from patients' serum at the indicated MOI in the presence of 4% PEG8000 for 24 hours. Input human serum volume was adjusted to the same between HBV from patients with or without NA therapy using serum from the healthy donor. Infected cultures were washed with PBS four times and maintained in fresh PHH maintenance medium with medium change at every other day.

TABLE 1. Clinical Information of the Pregnant Women Cohort Under LdT Therapy (n = 10)

Characteristics		Values
Age (years)		31 (28-35)*
NAs		LdT
HBV DNA (log_{10} IU/mL)	Baseline	>8.23 (n = 9), 7.86 (n = 1)
	Treatment for 4-8 weeks	4.64 (4.43-5.10)*
	Treatment for 12 weeks (end point)	3.85 (3.47-4.22)*
	Post EoT 5-6 weeks	>8.23 (n = 7), 7.43 (n = 1), 7.44 (n = 1), 7.96 (n = 1)

*Median (interquartile range). The upper limit of quantification (ULoQ) is 1.7E+8 IU/mL (ULoQ = 8.23 log_{10}).
Abbreviation: EoT, end of treatment.

DETECTION OF HEPATITIS B SURFACE ANTIGEN AND HEPATITIS B e ANTIGEN

Media of HepG2-NTCP and PHH cultures were harvested at 2, 4, and 6 days post-HBV infection, and levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in culture media were detected by time-resolved fluoroimmunoassay, according to the manufacturer's instructions (PerkinElmer, Waltham, MA).

EXTRACTION OF HBV DNA AND RNA AND HBV-RNA REVERSE TRANSCRIPTION

The HBV DNA and RNA in the culture medium of HepAD38 cells and sera of patients were extracted

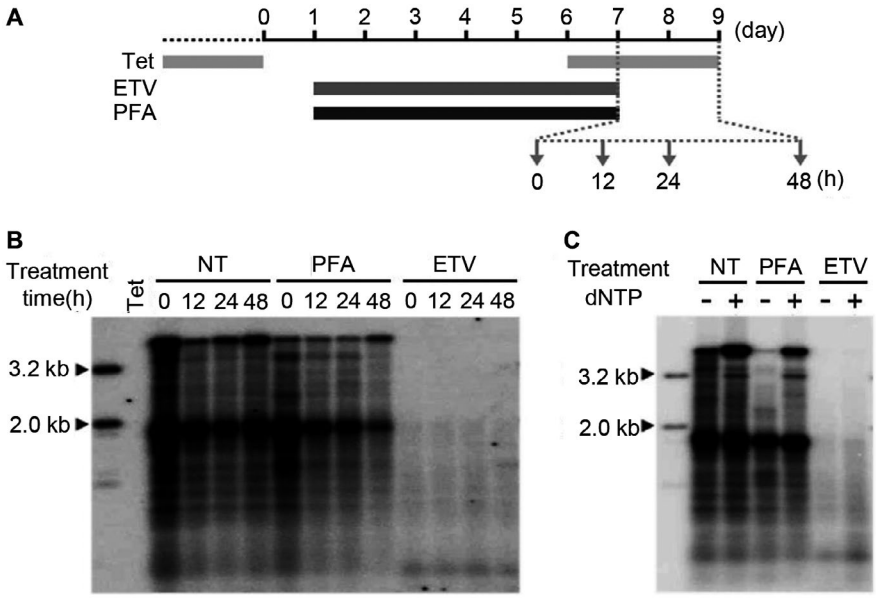


FIG. 4. The HBV DNA produced under NA treatment was irreversibly blocked for elongation by viral DNA polymerase. (A) Schematic presentation of the timeline of the experiment. Briefly, HepAD38 cells were maintained in culture in the presence of 0.1 μg/mL of tetracycline (tet+). Cells were seeded into 12-well plates and cultured in the absence of tet. One day later, cells were mock-treated (NT) or treated with 2 mM of PFA or 0.1 μM of ETV. At day 6 postseeding, tet was added back to culture media to stop pgRNA transcripton from transgene. PFA and ETV treatment were stopped 1 day later (day 7). Cells were harvested at 0, 12, 24, and 48 hours post-PFA or ETV treatment. (B) HBV core DNA was detected by Southern blotting hybridization assay with α-^{32}P-UTP–labeled full-length plus-strand HBV RNA. (C) Intracellular HBV capsids were purified by sucrose gradient centrifugation from the lysates of cells harvested at day 7. An endogenous DNA polymerase assay was performed in the absence or presence of dNTP for 16 hours. HBV DNA were extracted and detected by Southern blotting hybridization with α-^{32}P-UTP–labeled full-length plus-strand HBV RNA.

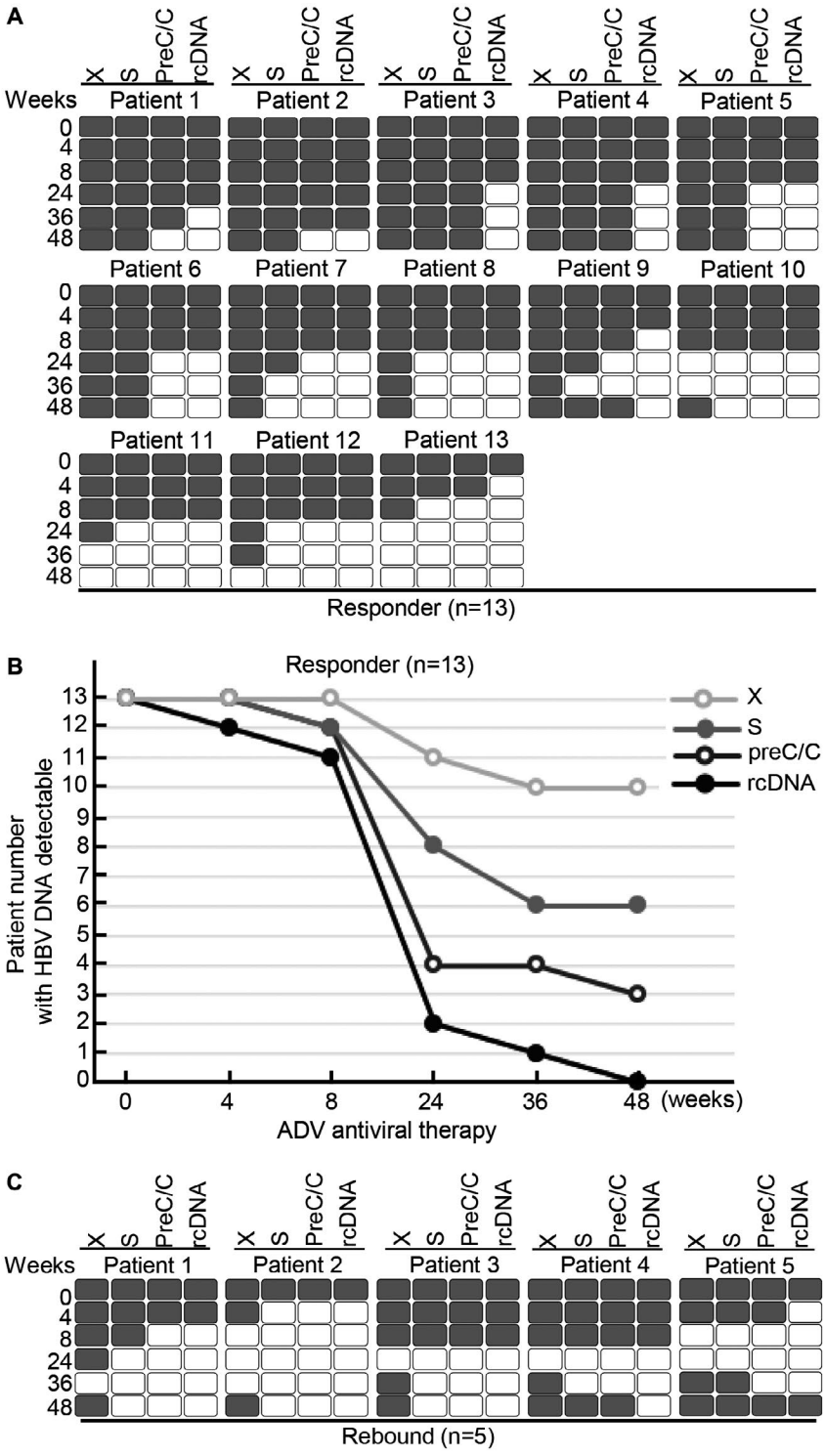


FIG. 5. Dynamics of HBV DNA in the serum of patients under ADV therapy. The detectability of HBV rcDNA and three different regions (X, S, and PreC/C) of HBV DNA in the serial serum samples from patients in the response group (n = 13; A) and rebound group (n = 5; C) by qPCR assays. Appropriate repetitive water samples as negative control were included in the qPCR assay of each region. Sample CT values less than water were scored as positive (solid), whereas CT values larger than any water sample were scored as negative (hollow). (B) Total numbers of patients with detectable levels of HBV DNA with each of the primer pairs in the response group at the indicated time points of therapy are presented.

using the EasyPure Viral RNA Kit (TransGen Biotech, Beijing, China), according to the manufacturer's instructions. For HBV-RNA extraction, the products were further treated with DNase I (Thermo Fisher Scientific, Waltham, MA). Isolated HBV RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit with random primers for RT-PCR (Roche, Basel, Switzerland), in accord with the manufacturer's instructions.

QUANTIFICATION OF HBV DNA AND RNA

HBV-DNA and RNA levels in the culture medium of HepAD38 cells and sera of patients were detected by qPCR in the LightCycler 480 II Real-time PCR Detection System (Roche, Mannheim, Germany) with a SYBR Green method. The four pairs of primers used to detect different regions of HBV DNA and RNA are as follows: rcDNA-F(1778-1798 nt): 5′-GGAGGCTGTAGGCATAAATTGG-3′, rcDNA-R(1883-1862 nt): 5′-CACAGCTTGGAGG CTTGAAC-3′; PreC/C-F(2299-2319 nt): 5′-AGA CCACCAAATGCCCCTATC-3′, PreC/C-R(2397-2378 nt): 5′-TCTGCGAGGCGAGGGAGTTC-3′; S-F(303-322 nt): 5′-TGGCCAAAATTCGCAGTC CC-3′, S-R(448-425 nt): 5′-GAAGAACCAACAA GAAGATGAGGC-3′; X-F(1608-1628 nt): 5′-CA TGGARACCACCGTGAACG, X-R(1800-1776 nt): 5′-CCAATTTATGCCTACAGCCTCCT-3′. The serial dilutions of pBB4.5-1.2×HBV plasmid were used as standards of quantification. The qPCR reaction mixture (20 μL) contained 10 μL of 2× mix (LightCycler 480 SYBR green Master; Roche), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 1 μL of DNA (for DNA detection) or 1 μL of complementary DNA template (for RNA detection), and 7 μL of double-distilled water (ddH_2O). The reaction mixture was denatured at 95°C for 5 minutes, followed by 45 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

SOUTHERN AND NORTHERN BLOTTING HYBRIDIZATION

Intracellular viral core DNA or encapsidated RNA were extracted as described.[28] Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY) by following the manufacturer's directions. HBV DNA and RNA were resolved by electrophoresis in agarose gel and transferred onto a Hybond-XL membrane. The membrane was probed with uridine 5′-[alpha-32P] triphosphate tetra(triethylammonium) salt (α-^{32}P-UTP)–labeled minus-strand (for Southern blotting) or plus-strand (for northern blotting) specific full-length HBV riboprobe.[28]

ENDOGENOUS DNA POLYMERASE ASSAY

An endogenous DNA polymerase assay was described.[28] Briefly, the reaction mixture was assembled with 20 μL of HBV virion (or nucleocapsids) preparation, 25 μL of 2× endogenous DNA polymerase reaction buffer, and a 0.1-mM final concentration of dNTP. Water was added to bring the reaction volume to 50 μL. After incubation at 37°C for an indicated period, viral DNA were extracted and resolved in 1.5% agarose gel and transferred onto a Hybond-XL membrane. The membrane was probed with α-^{32}P-UTP–labeled minus-strand specific full-length HBV riboprobe.

STATISTICAL ANALYSES

For statistical analysis, a two-tailed Student t test was performed using the Statistical Analysis System software (GraphPad Prism; GraphPad Software Inc., La Jolla, CA). In all cases, a *P* value of <0.05 was considered significant. Data were presented as the mean and SD from at least three independent experiments.

Results

HBV VIRIONS PRODUCED UNDER NA TREATMENT WERE MAINLY DISABLED TO INFECT HepG2-NTCP CELLS

In order to test the infectivity of virions produced under NA treatment, HepAD38 cells cultured in the absence of tetracycline were treated with ETV or control solvent (DMSO; Fig. 1A). HBV virions were prepared from the culture media by polyethylene glycol precipitation. To ensure that the same amount of virions was used for *in vitro* infection assays, viral titers of ETV- and DMSO-treated HBV virion preparations were determined by qPCR quantification targeting the

X region of viral DNA (Fig. 1A), which were further confirmed by a western blotting assay demonstrating that equal amounts of HBV core (HBc) protein presented in ETV- and DMSO-treated HBV virion preparations (Fig. 1B). HepG2-NTCP cells, which express NTCP in a doxycycline (Dox)-inducible manner (Fig. 1C), were infected by virions prepared from ETV- or DMSO-treated HepAD38 cultures at MOI of 500, 1,000, and 2,000 genome-equivalents. Gradual increases of HBeAg and HBsAg were observed in a time- and MOI-dependent manner in the media of cells infected with virions produced by DMSO-treated HepAD38 cells (Fig. 1D,E). In contrast, no or only trace amounts of HBeAg and HBsAg were detectable in the media of cells infected with virions produced by ETV-treated HepAD38 cells. These results thus suggested that the virions produced by ETV-treated HepAD38 are mainly not infectious. In agreement with this notion, an obvious increase of HBV-DNA levels from day 4 to day 6, an indication of active viral DNA replication and secretion of progeny virions, was observed in the culture media of cells infected with virions from DMSO-treated, but not ETV-treated, HepAD38 cells (Fig. 1F). Moreover, to determine the half maximal inhibitory concentration (IC_{50}) of ETV in inhibiting progeny HBV infectivity, HepG2-NTCP cells were infected with HBV virions prepared from HepAD38 cells treated with ETV at a serial concentration of 100, 300, 600, 1,200, and 2,500 nM (Fig. 1G). The IC_{50} was calculated based on the HBeAg levels at 2 days postinfection. The results showed that ETV treatment dramatically reduced the infectivity of progeny HBV virions in a dose-dependent manner (Fig. 1G). The calculated IC_{50} of ETV in inhibiting progeny HBV infectivity was approximately 350.5 ± 64.19 nM (Fig. 1H).

HBV VIRIONS PRODUCED UNDER NA TREATMENT WERE MAINLY DISABLED TO INFECT PRIMARY HUMAN HEPATOCYTES

Next, we tested the infectivity of virions prepared either from ETV (2.5 μM)/DMSO–treated HepAD38 cells or from sera of a CHB patient with or without NA therapy by using PHHs. Consistent with the results obtained from HepG2-NTCP cells, a gradual increase of HBeAg and HBsAg were observed in a time- and MOI-dependent manner in the media of PHH infected with virions produced by DMSO-treated HepAD38 cells (Fig. 2A,B), whereas there were no or only trace HBeAg or HBsAg could be detected in the media of PHH infected with virions produced by ETV (2.5 μM)–treated HepAD38 cells. Then, PHHs were infected with HBV from the treatment-naïve patients, and the patient underwent TDF therapy for 4 weeks. PCR-based direct sequencing assay indicated all patients were infected with genotype C HBV (Supporting Fig. S1). As expected, gradual increase of HBeAg was only detected in the media of PHHs infected with virions from treatment-naïve patients, but not from the TDF-treated patient (Fig. 2C). The continuous decline of HBsAg also suggested that the HBV from the TDF-treated patient was not infectious to PHHs (Fig. 2D). The high HBsAg titers at 2 days postinfection is most likely attributable to the input HBV interference.

HBV DNA PRODUCED UNDER NA TREATMENT WERE PREDOMINANTLY 3′ TRUNCATED, MINUS-POLARITY, SINGLE-STRANDED DNA

Given that NAs act as chain terminators upon incorporation into the growing HBV-DNA chain,[22] it is reasonable to postulate that under NA treatment, the *de novo* synthesis of nascent HBV-DNA chain would be prematurely terminated at any site during the process of reverse transcription. This hypothesis predicts that the *de novo* synthesis of HBV minus-strand DNA under NA treatment will be terminated in a sequentially decreased frequency from the reverse-transcription initiation region (DR1/X gene), S gene region, to the PreC/C gene region (Fig. 3A-C). Moreover, in the case that the HBV replication escapes NA incorporation during minus-strand DNA synthesis, NA treatment still had a chance to terminate plus-strand DNA elongation and rcDNA formation (Fig. 3B). On the other hand, the pgRNA would be digested sequentially from the DR1 region toward its 5′ terminus during the process of minus-strand DNA synthesis by RNase H.[8] Therefore, NA treatment will result in accumulation of 3′ truncated pgRNA in nuclecapsids or secreted virions, and the abundance of detectable pgRNA sequence gradually increased from the DR1/X gene region toward its 5′ terminus (Fig. 3C). In addition to pgRNA, HBV-RNA species that are smaller than pgRNA and variable in length can also be detected in nucleocapsids.[24,29]

Consistent with our previous observation,[5] inhibition of *de novo* HBV-DNA synthesis by ETV or foscarnet (phosphonoformic acid; PFA) increased the accumulation of encapsidated pgRNA, as compared to that of mock-treated control, because of the premature termination of minus-strand HBV DNA synthesis by ETV or PFA (Fig. 3D).

In order to obtain evidence supporting that HBV-DNA synthesis is prematurely terminated by NA treatment at the predicted frequency along the viral DNA and pgRNA, three pairs of PCR primers were designed to specifically quantify HBV DNA corresponding to the PreC/C, S, and X regions of the newly synthesized minus-strand DNA and RNase H–digested pgRNA (Fig. 3A). An additional pair of primers was used for specifically measuring rcDNA.[30] The qPCR reaction conditions had been optimized to make sure of similar amplification efficiency for each set of primers before the experiment was being conducted (Supporting Fig. S2). The lower limit of detection (LLoD) and lower limit of quantification of the four amplification systems were similar and all ranged from 3 to 3.25 $\log_{10}$ copies/mL and approximately 4 $\log_{10}$ copies/mL, respectively (Supporting Fig. S3). HBV DNA and RNA in sera specimens collected from a cohort of 10 pregnant women were quantified by the four PCR assays in parallel. As previously described, all these individuals had high levels of HBV DNA and received LdT therapy to prevent the mother-to-child transmission.[24] Briefly, the serum specimens were collected at four time points: pretreatment (baseline), 4-8 weeks of treatment and 12 weeks of treatment at perinatal period, as well as 5-6 weeks after delivery with LdT withdraw. Levels of HBV DNA, determined by the four pairs of primers, showed no difference at baseline (Fig. 3E). However, accompanying the significant decline of HBV-DNA load after LdT treatment at 4-8 weeks and 12 weeks, the magnitude of DNA decline from the highest to the lowest was rcDNA, PreC/C region, S region, and X region, with statistical differences between them at 4-8 weeks (P = 0.0029 for X vs. rcDNA; P = 0.0185 for S vs. rcDNA) and 12 weeks (P = 0.0007 for X vs. rcDNA; P = 0.0068 for S vs. rcDNA; P = 0.0355 for X vs. S) of LdT treatment (Supporting Fig. S4). However, the differences disappeared and the levels of HBV DNA rebounded to baseline level at 5-6 weeks after LdT withdrawal (Fig. 3E). Meanwhile, HBV-RNA levels in sera of patients showed a tendency of gradually increase, from X region, S region, to PreC/C region, even at baseline before LdT treatment (Fig. 3F). The significant increase of HBV RNA in the X region after LdT treatment (P = 0.0244; Supporting Fig. S5) is consistent with the arrest of minus-strand DNA synthesis at the early stage and accumulation of slightly shorter than full-length pgRNA in nucleocapsids.[31]

IFN-α controls HBV infection mainly by direct suppression of HBV replication by induction of antiviral immune response. It is thus anticipated that IFN-α therapy does not cause differential reduction of HBV-DNA species in sera of patients. Indeed, serum HBV-DNA quantification of rcDNA and three different regions of viral DNA among the 24 patients recieved Peg-IFN-α-2a/b therapy revealed similar levels of HBV DNA at baseline, after receiving 48 weeks of Peg-IFN-α-2a/b treatment and at week 24 posttherapy follow-up (Supporting Fig. S6). Taken together, the above results imply that unlike IFN-α therapy, NA therapy arrests HBV-DNA synthesis and results in the production of virion-like particles containing 3′ truncated pgRNA and variable length of minus-strand DNA in cultured cells and *in vivo* in the serum of patients.

HBV-DNA SYNTHESIS WAS IRREVERSIBLY TERMINATED BY NA TREATMENT

To confirm whether the replication-arrested viral DNA accumulated in NA-treated cells is irreversibly terminated, the following two experiments were performed using HepAD38 cells. HBV-DNA replication was arrested by culturing HepAD38 cells in medium without tetracycline, but containing control solvent, ETV, or PFA for 6 days (Fig. 4A). Tetracycline was then added back to culture media to stop pgRNA transcription from integrated transgene. Twenty-four hours later, cells were cultured in the presence of tetracycline and absence of ETV or PFA to allow viral DNA replication to resume. Cells were harvested at 0, 12, 24, and 48 hours after ETV or PFA withdrawal for analyses of HBV DNA. Upon removal of polymerase inhibitor from culture medium (day 7, 0-hour time point), the arrested minus-strand DNA gradually decreased as rcDNA increased from 0 to 48 hours in PFA-treated cells (Fig. 4B). On the contrary, ETV treatment arrested HBV DNA at the early stage of minus-strand DNA and thus only accumulated short

minus-strand DNA. Upon removal of ETV, the short minus-strand DNA was not further elongated within 48 hours of culture.

To further validate these results, HBV capsids were purified from the cytoplasmic fraction of ETV- or PFA-treated HepAD38 cells and an endogenous DNA polymerase assay was performed *in vitro*. Southern blotting analysis of HBV DNA indicated that addition of dNTP efficiently extended PFA-arrested minus-strand DNA to rcDNA, but failed to significantly elongate the ETV-arrested short minus-strand DNA (Fig. 4C). These results thus indicate that as anticipated, whereas PFA reversibly inhibited HBV DNA synthesis,[28] ETV, and other NAs by inference, it irreversibly terminated HBV-DNA synthesis by incorporating into growing nascent HBV-DNA chains.

SEQUENTIAL DISAPPEARANCE OF SERUM HBV DNA IN PATIENTS AFTER RECEIVING NA TREATMENT

In order to further investigate the dynamic changes of HBV DNA in the peripheral blood of patients under NA treatment, a cohort of 18 CHB patients treated with ADV for 48 weeks were enrolled in this study. According to their clinical therapy characteristics,[26] the patients were categorized into two groups. The response group (n = 13) includes individuals with virological response or partial virological response, and the rebound group (n = 5) includes patients who experienced virological breakthrough.[32] The results showed that after initiation of treatment, rcDNA disappearance occurred first, followed by the sequential disappearance of HBV DNA corresponding to the regions of PreC/C, S, and X gene (Fig. 5A,B). Specifically, rcDNA became undetectable in 1 patient (#13) after 4 weeks of treatment and in all the patients after 48 weeks of treatment in the response groups. Interestingly, the X region DNA was usually the last one to lose, but the first one to rebound, in the rebound group (Fig. 5C), because the synthesis of the X region of HBV DNA was foremost to escape NA irreversible termination. This dynamic feature of HBV DNA further supports the hypothesis that NA treatment prematurely terminates HBV-DNA synthesis and results in the secretion of virions containing short minus-strand DNA. Moreover, given that the undetectable level of serum HBV DNA is an important indicator of virological response to antiviral therapy, the observed difference in detection of different regions of HBV DNA in NA-treated patients strongly argues that PCR assays specifically measure the X region of HBV DNA should be used in clinical practice to monitor NA treatment response, because it becomes undetectable last, but rebounds first, upon breakthrough.

Discussion

In today's clinic practices, NA therapy is often preferred, considering the relative safety, tolerability, and oral convenience. As the substrate analogues of viral DNA polymerase, NAs can specifically compete with their corresponding endogenous nucleotides for incorporation into the growing nascent viral DNA chain and nonspecially inhibit HBV-DNA chain elongation. As a result, the progeny HBV virions produced under NA treatment may contain only incomplete, dead-end HBV genomic DNA and are thus mainly noninfectious. In addition, previous studies also revealed the lack of preS1 NTCP-binding domain in the RNA virion-like particles produced under the NA treatment.[24,29] In this study, we demonstrated that NAs inhibit HBV replication in cultured hepatocytes, PHH, as well as *in vivo* in patients with the anticipated mode of action.

Our findings are of important clinical implications. First, HBV-DNA level in blood, or viral load, has been used as the standard biomarker to evaluate the virological response of NA therapies.[32-34] In treatment-naïve CHB patients, quantification of viral load by amplification of different regions of HBV DNA yields similar results (Fig. 2). However, because of the premature termination of HBV-DNA synthesis in NA-treated patients, viral load could vary among the assays that detect different regions of HBV DNA. Indeed, in NA-treated patients, the values of viral load obtained by qPCR amplification of the reverse-transcription initiation region (in X gene), S gene region, PreC/C region, to the rcDNA-specific region sequentially reduce and differ by more than 1 $\log_{10}$ (Fig. 2). Therefore, the reported "HBV load" in NA-treated subjects may vary from kit to kit that detects different regions of HBV DNA. Furthermore, as expected, but different from a recent report,[35] the levels of serum HBV RNA are highest in the PreC/C region and declined from the S region to X region. Notably, HBV-RNA level may also

be affected by the RNA splicing-variants given that some of them lack the S region, particularly after NA treatment.[24,29] Therefore, in order to more accurately monitor viral level and determine the residual activity of HBV replication under NA therapies, we strongly recommend to measure viral load and serum HBV RNA by qPCR assays that detect the X gene region and PreC/C region, respectively. Moreover, because of the progeny HBV DNA under NA treatment are primarily dead-end short minus-strand DNA, the "viral load" under this condition does not truly reflect the level of infectious virions.

Second, it is well known that persistence of cccDNA is the major determinant of chronicity in patients with CHB, and elimination of the cccDNA reservoir is thought to be fundamental to resolve HBV infection.[36,37] Recently, Allweiss et al. reported that immune-mediated destruction of HBV-infected hepatocytes and the compensatory hepatocyte division resulted in dilution of the cccDNA pool.[38] Because of the irreversible termination of HBV-DNA synthesis and production of noninfectious virions, NA treatment not only inhibits intracellular cccDNA amplification, but also inhibits the replenishment of the cccDNA pool by reinfection as well as the spreading of HBV infection to naïve hepatocytes.[39] It is thus reasonable to predict that combining immunotherapies that destroy infected hepatocytes and accelerate hepatocyte turnover with NA therapy that inhibits cccDNA replenishment and reinfection of cccDNA-free hepatocytes may result in cccDNA clearance, particularly after prolonged NA treatment.

Finally, accumulating evidence suggests that the risk of mother-to-child transmission can be dramatically decreased by NA treatment of pregnant women with high HBV-DNA load during the perinatal period.[32-34,40] Our current study implies that this should be achieved not only by reducing viral load, but also reducing the infectivity of progeny virions. We wondered, in resource-limited settings, whether NA treatment of pregnant women during the perinatal period in combination with universal vaccination of the newborns would effectively prevent HBV vertical transmission among pregnant women with high viral loads during the perinatal period. In addition, several recent studies seek to minimize the dose or duration of hepatitis B immune globulin (HBIG) for prevention of HBV recurrence after liver transplantation.[41-43] With the high potent and low resistant NAs, such as ETV and TDF, stopping HBIG and continuing antiviral monotherapy could be used as an effective prophylaxis strategy for many, if not most, patients.[44,45] Our finding that NA treatment results in the production of noninfectious virions provides a strong support for HBIG-free prophylaxis of recurrent HBV infection in the setting of liver transplantation. It also implies that chronically HBV-infected individuals under NA therapy may have less opportunity to transmit HBV to their sexual and other intimate contacts.

In conclusion, the current study suggest that HBV virions produced under NA treatment are predominantly in replication-deficient form, attributable to the irreversible termination and truncation of nascent viral genomes. These findings indicate that in patients under NA treatment, the "viral load" measured by serum HBV quantification would actually be part of the replication-deficient HBV-DNA fragments.

REFERENCES

1) Poh Z, Goh BB, Chang PE, Tan CK. Rates of cirrhosis and hepatocellular carcinoma in chronic hepatitis B and the role of surveillance: a 10-year follow-up of 673 patients. Eur J Gastroenterol Hepatol 2015;27:638-643.
2) Gish RG, Given BD, Lai CL, Locarnini SA, Lau JY, Lewis DL, Schluep T. Chronic hepatitis B: virology, natural history, current management and a glimpse at future opportunities. Antiviral Res 2015;121:47-58.
3) Hutin YJ, Bulterys M, Hirnschall GO. How far are we from viral hepatitis elimination service coverage targets? J Int AIDS Soc 2018;21(Suppl 2):e25050.
4) Summers J, O'Connell A, Millman I. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proc Natl Acad Sci U S A 1975;72:4597-4601.
5) **Wang J**, **Shen T**, **Huang X**, **Kumar GR**, Chen X, Zeng Z, et al. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. J Hepatol 2016;65:700-710.
6) Bai L, Zhang X, Li W, Wu M, Liu J, Kozlowski M, et al. Extracellular HBV RNAs are heterogeneous in length and circulate as capsid-antibody-complexes in addition to virions in chronic hepatitis B patients. J Virol 2018;92:e00798-18.
7) **Yan H**, **Zhong G**, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 2012;1:e00049.
8) Summers J, Mason WS. Replication of the genome of a hepatitis B–like virus by reverse transcription of an RNA intermediate. Cell 1982;29:403-415.
9) Urban S, Schulze A, Dandri M, Petersen J. The replication cycle of hepatitis B virus. J Hepatol 2010;52:282-284.
10) Wang GH, Seeger C. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell 1992;71:663-670.
11) Weber M, Bronsema V, Bartos H, Bosserhoff A, Bartenschlager R, Schaller H. Hepadnavirus P protein utilizes a tyrosine residue in the TP domain to prime reverse transcription. J Virol 1994;68:2994-2999.

12) Loeb DD, Hirsch RC, Ganem D. Sequence-independent RNA cleavages generate the primers for plus strand DNA synthesis in hepatitis B viruses: implications for other reverse transcribing elements. EMBO J 1991;10:3533-3540.
13) Nassal M, Rieger A. A bulged region of the hepatitis B virus RNA encapsidation signal contains the replication origin for discontinuous first-strand DNA synthesis. J Virol 1996;70:2764-2773.
14) Haines KM, Loeb DD. The sequence of the RNA primer and the DNA template influence the initiation of plus-strand DNA synthesis in hepatitis B virus. J Mol Biol 2007;370:471-480.
15) Yang W, Summers J. Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor. J Virol 1999;73:9710-9717.
16) Gupta N, Goyal M, Wu CH, Wu GY. The molecular and structural basis of HBV-resistance to nucleos(t)ide analogs. J Clin Transl Hepatol 2014;2:202-211.
17) Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999; 341:1256-1263.
18) Marcellin P, Chang TT, Lim SG, Sievert W, Tong M, Arterburn S, et al. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. Hepatology 2008;48:750-758.
19) **Marcellin P**, **Heathcote EJ**, Buti M, Gane E, de Man RA, Krastev Z, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N Engl J Med 2008; 359:2442-2455.
20) Seto WK, Hui AJ, Wong VW, Wong GL, Liu KS, Lai CL, et al. Treatment cessation of entecavir in Asian patients with hepatitis B e antigen negative chronic hepatitis B: a multicentre prospective study. Gut 2015;64:667-672.
21) De Clercq E. Tenofovir alafenamide (TAF) as the successor of tenofovir disoproxil fumarate (TDF). Biochem Pharmacol 2016;119:1-7.
22) Fung J, Lai CL, Seto WK, Yuen MF. Nucleoside/nucleotide analogues in the treatment of chronic hepatitis B. J Antimicrob Chemother 2011;66:2715-2725.
23) Langley DR, Walsh AW, Baldick CJ, Eggers BJ, Rose RE, Levine SM, et al. Inhibition of hepatitis B virus polymerase by entecavir. J Virol 2007;81:3992-4001.
24) Wang J, Sheng Q, Ding Y, Chen R, Sun X, Chen X, et al. HBV RNA virion-like particles produced under nucleos(t)ide analogues treatment are mainly replication-deficient. J Hepatol 2018;68:847-849.
25) 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.
26) Zhao XL, Yang JR, Lin SZ, Ma H, Guo F, Yang RF, et al. Serum viral duplex-linear DNA proportion increases with the progression of liver disease in patients infected with HBV. Gut 2016;65:502-511.
27) **Wang J**, **Chen R**, **Zhang R**, Ding S, Zhang T, Yuan Q, et al. The gRNA-miRNA-gRNA ternary cassette combining CRISPR/Cas9 with RNAi approach strongly inhibits hepatitis B virus replication. Theranostics 2017;7:3090-3105.
28) Guo F, Zhao Q, Sheraz M, Cheng J, Qi Y, Su Q, et al. HBV core protein allosteric modulators differentially alter cccDNA biosynthesis from de novo infection and intracellular amplification pathways. PLoS Pathog 2017;13:e1006658.
29) Sommer G, Heise T. Posttranscriptional control of HBV gene expression. Front Biosci 2008;13:5533-5547.
30) Liu Y, Zeng W, Xi J, Liu H, Liao H, Yu G, et al. Over-gap PCR amplification to identify presence of replication-competent HBV DNA from integrated HBV DNA: an updated occult HBV infection definition. J Hepatol 2019;70:557-559.
31) Zhang P, Liu F, Guo F, Zhao Q, Chang J, Guo JT. Characterization of novel hepadnaviral RNA species accumulated in hepatoma cells treated with viral DNA polymerase inhibitors. Antiviral Res 2016;131:40-48.
32) European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;67:370-398.
33) Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, et al. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int 2016;10:1-98.
34) Terrault NA, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH; American Association for the Study of Liver Diseases. AASLD guidelines for treatment of chronic hepatitis B. Hepatology 2016;63:261-283.
35) Butler EK, Gersch J, McNamara A, Luk KC, Holzmayer V, de Medina M, et al. Hepatitis B virus serum DNA and RNA levels in Nucleos(t)ide analog-treated or untreated patients during chronic and acute infection. Hepatology 2018;68:2106-2117.
36) Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med 2004;350: 1118-1129.
37) Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015;64:1972-1984.
38) Allweiss L, Volz T, Giersch K, Kah J, Raffa G, Petersen J, et al. Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. Gut 2018;67:542-552.
39) Ko C, Chakraborty A, Chou WM, Hasreiter J, Wettengel JM, Stadler D, et al. Hepatitis B virus genome recycling and de novo secondary infection events maintain stable cccDNA levels. J Hepatol 2018;69:1231-1241.
40) Lu Y, Zhu FC, Liu JX, Zhai XJ, Chang ZJ, Yan L, et al. The maternal viral threshold for antiviral prophylaxis of perinatal hepatitis B virus transmission in settings with limited resources: a large prospective cohort study in China. Vaccine 2017;35:6627-6633.
41) Schiff E, Lai CL, Hadziyannis S, Neuhaus P, Terrault N, Colombo M, et al. Adefovir dipivoxil for wait-listed and post-liver transplantation patients with lamivudine-resistant hepatitis B: final long-term results. Liver Transpl 2007;13:349-360.
42) Yoshida H, Kato T, Levi DM, Regev A, Madariaga JR, Nishida S, et al. Lamivudine monoprophylaxis for liver transplant recipients with non-replicating hepatitis B virus infection. Clin Transplant 2007;21:166-171.
43) Liaw YF, Sheen IS, Lee CM, Akarca US, Papatheodoridis GV, Suet-Hing Wong F, et al. Tenofovir disoproxil fumarate (TDF), emtricitabine/TDF, and entecavir in patients with decompensated chronic hepatitis B liver disease. Hepatology 2011;53:62-72.
44) Gane EJ, Patterson S, Strasser SI, McCaughan GW, Angus PW. Combination of lamivudine and adefovir without hepatitis B immune globulin is safe and effective prophylaxis against hepatitis B virus recurrence in hepatitis B surface antigen-positive liver transplant candidates. Liver Transpl 2013;19:268-274.
45) Fox AN, Terrault NA. The option of HBIG-free prophylaxis against recurrent HBV. J Hepatol 2012;56:1189-1197.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30844/suppinfo.