Supporting Information

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SI Materials and Methods

Purification and Analysis of HBV DNA from Core Particles. To purify HBV DNA from intracellular core particles, transfected cells were washed once with ice-cold PBS and lysed in 50 mmol Tris-HCl, pH 7.4, 1 mmol EDTA, and 1% Nonidet P-40 (lysis buffer A). Nuclei were pelleted by centrifugation for 1 min at 14,000 rpm. The supernatant was adjusted to 100 mmol $MgCl_2$ and treated with 100 μg/mL DNase I for 30 min at 37 °C. The reaction was stopped by addition of EDTA to a final concentration of 25 mmol. Protein were digested with 0.5 mg/mL proteinase K and 1% SDS for 2 h at 50 °C. Nucleic acids were purified by phenol/chloroform (1:1) extraction and ethanol precipitation adding glycogen and examined by Southern blot analysis (1).

Quantification of HBV cccDNA by Real-Time PCR from HBV-Transfected Cells. HepG2 cells were collected at the indicated times after transfection, resuspended in lysis buffer A, and incubated 10 min at 4 °C. Lysates were centrifuged 1 min at 13,000 rpm, pelleted nuclei resuspended in other lysis buffer B (10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.5% SDS, and 0.5 mg/mL proteinase K), and incubated overnight at 37 °C. Nucleic acids were purified by phenol/chloroform (1:1) extraction and ethanol precipitation. Aliquots (500 ng) of each extracted DNA were treated for 45 min at 37 °C with 10 U Plasmid safe DNase I (Epicentre). DNase was inactivated by incubating the reactions for 30 min at 70 °C. Real-time PCR experiments were performed in a Light-Cycler (Roche Diagnostics) using a 20-μL reaction volume containing 20 ng DNA, 3 mmol/L $MgCl_2$, 0.5 mmol/L forward and reverse primers, 0.2 mmol/L 3′-fluorescein (FL)-labeled probe, and 0.4 mmol/L 5′-Red640 (R640)-labeled probe. Forward and reverse primers were 5′-CTCCCCGTCTGTGCCTTCT-3′ (NCCC1 nt 1548–1566) and 5′-GCCCCAAAGCCACCCAAG-3′ (CCCAS2 nt 1903–1886), respectively, whereas the hybridization probes were 5′-GTTCACGGTGGTCTCCATGCAACGT-FL-3′ and 5′-R640-AGGTGAAGCGAA GTGCACACG-GACC-3′, respectively. Amplification was performed as follows: 95 °C for 10 min then 45 cycles of 95 °C for 10 s, 58 °C for 5 s, 63 °C for 10 s, and 72 °C for 20 s. The efficacy of DNase treatment in the elimination of OC and SS forms of HBV DNA before PCR was confirmed by the abrogation of the PCR amplification of HBV DNA extracted from cytoplasmic viral particles by the nonselective HBV oligonucleotide primers target the HBs ORF (2). β-Globin amplification was performed using the Light-Cycler β-globin control kit (Roche Diagnostics). Serial dilutions of a plasmid containing a monomeric genotype D HBV insert (Clonit S.r.l.) were used as quantification standards.

Isolation of HBV cccDNA from Liver Tissues. To isolate and analyze HBV cccDNA from the liver of a chronic hepatitis B patient (Fig. S1), the liver biopsy specimen was homogenized with an Ultra Turrax T25 device in 500 μL lysis buffer A at 4 °C and centrifuged for 10 min at 10,000 rpm at 4 °C (HB-4 rotor; Sorvall). Nuclei were then treated with 500 mL lysis buffer (6% SDS, 100 mmol NaOH), and the reaction was mixed thoroughly and incubated for 30 min at 37 °C. After neutralization with 3 mol potassium acetate (pH 4.8), lysates were cleared for 20 min at 10,000 rpm at 4 °C. HBV DNA was extracted from the supernatant with phenol/chloroform, precipitated with ethanol in the presence of 10 μg tRNA, and dissolved in 50 μL 10 mmol Tris-HCl (pH 7.5) and 1 mmol EDTA. Twenty microliters of the isolated low-molecular-weight DNA samples were used for HBV-specific Southern blot analysis (1).

Northern Blot RNA Analysis. For Northern blot analysis (Fig. S1) 25 μg total RNA per sample were separated on a 1% formaldeide-agarose gel and blotted onto Zeta-Probe GT membranes (Bio-Rad Laboratories). Radioactive probes were prepared by random priming protocol, using either full-length HBV DNA or 18.S cDNA templates and ^{32}P a-dCTP (Amersham). After hybridization, the membrane was washed and exposed to X-Omat film (Kodak) at −80 °C.

cccDNA ChIP Assays. The cccDNA ChIP assays were performed as described (2) with minor modifications. Briefly, at the indicated times after transfection with linear monomeric HBV genomes, HepG2 cells were resuspended in 1 mL lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA, and 100 mM NaCL) and incubated for 10 min at 4 °C. Lysates were the centrifuged at 13,000 rpm for 2 min, supernatants were removed, and nuclei were fixed in 1% formaldehyde for 20 min at 4 °C. Isolated cross-linked nuclei were then extracted with a 20 mM Tris, pH 8.0, 3 mM $MgCl_2$, 20 mM KCl buffer supplemented with a protease inhibitors mixture (PIC; Sigma), pelleted by microcentrifugation, and lysed by incubation in PIC-supplemented SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). Chromatin solutions were sonicated for 10 pulses of 45 s at 80% power to generate 300- to 400-bp DNA fragments using a Bioruptor apparatus (Diagenode). HBV cccDNA has been shown to be resistant to sonication, and intact cccDNA species can be detected by Southern blot analysis even after extensive (15–20 pulses) and prolonged sonication (2). After centrifugation, supernatants were diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-chloride, pH 8.1, 167 mM NaCl) containing PIC (Sigma) precleared with blocked protein G-Plus (Pierce). Chromatin was then subjected to immunoprecipitation for 14–16 h at 4 °C using antibodies specific to H4 (catalog no. 07–108, rabbit polyclonal IgG recognizing un-modified human 10 K_d H4 histone; Upstate), AcH4 (catalog no. 06–599, rabbit polyclonal IgG recognizing histone H4 tetra-acetylated at lysine 6, 9, 13, and 17; Upstate), AcH3 (catalog no. 06–599, rabbit polyclonal IgG recognizing histone H3 di-acetylated at lysine 9 and 14; Upstate), p300 (catalog no. sc 585; Santa Cruz Biotechnology), CBP (catalog no. 06–294; Upstate), PCAF (catalog no. sc 8999; Santa Cruz Biotechnology), E2F1 (catalog no. sc 193; Santa Cruz Biotechnology), hSirt1 (catalog no. sc 15404; Santa Cruz Biotechnology), HDAC1 (catalog no. 06–720; Upstate), and HBV-HBx (catalog no. MA1–081; Affinity BioReagents). Immunoprecipitations with relevant nonspecific immunoglobulins (Santa Cruz Biotechnology) were included in each experiment as a negative control. Immunoprecipitated chromatins were processed and analyzed by PCR and real-time PCR using cccDNA selective primers and probes (2, 3).

1. Pollicino T, et al. (2004) Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology* 126:102–110.
2. Pollicino T, et al. (2006) Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology* 130:823–837.
3. Werle-Lapostolle B, et al. (2004) Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 126:1750–1758.

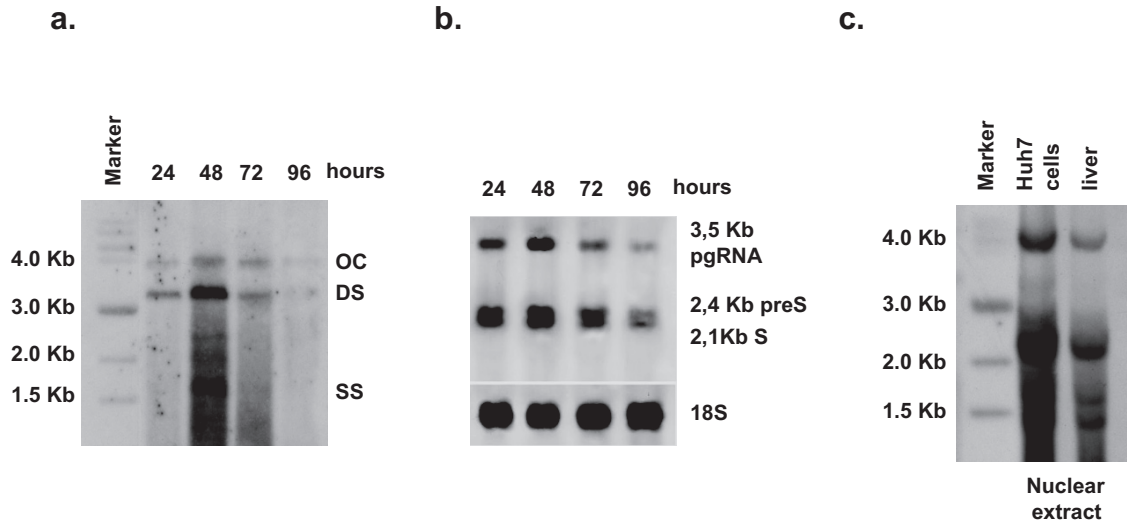

Fig. S1. Linear monomeric HBV genomes after transfection into HepG2 cells start a complete viral replication cycle. (*a*) Southern blot analysis of HBV replicative intermediates. Lanes correspond to DNA extracted from cytoplasmic HBV core particles derived from HepG2 cells that were transfected with 200 ng linear monomeric WT HBV genomes. Cells were harvested at the indicated time posttransfection. Membranes were exposed to an X-ray film for autoradiography at −80 °C for 24 h. (*b*) Northern blot analysis of HBV transcripts isolated from HepG2 cells 24, 48, 72, and 96 h after transfection with 200 ng monomeric linear HBV genomes. (*c*) Southern blot analysis of HBV cccDNA selectively extracted from HBV-transfected cells and from liver tissue of a chronic hepatitis B patient used as a positive control. OC, open circular duplex HBV DNA; DS, double-strand HBV DNA replicative intermediates; SS, single strand HBV DNA replicative intermediates.