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ORIGINAL ARTICLE

Replication of a chronic hepatitis B virus genotype F1b construct

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Abstract Genotype F is one of the less-studied genotypes of human hepatitis B virus, although it is widely distributed in regions of Central and South American. Our previous studies have shown that HBV genotype F is prevalent in Chile, and phylogenetic analysis of its full-length sequence amplified from the sera of chronically infected patients identified it as HBV subgenotype F1b. We have previously reported the full-length sequence of a HBV molecular clone obtained from a patient chronically infected with genotype F1b. In this report, we established a system to study HBV replication based on hepatoma cell lines transfected with full-length monomers of the HBV genome. Culture supernatants were analyzed after transfection and found to contain both HBsAg and HBeAg viral antigens. Consistently, fractionated cell extracts revealed the presence of viral replication, with both cytoplasmic and nuclear DNA intermediates. Analysis of HBV-transfected cells by indirect immunofluorescence or immunoelectron microscopy revealed the expression of viral antigens and cytoplasmic viral particles, respectively. To test the functionality of the ongoing viral replication further at the level of chromatinized cccDNA, transfected cells were treated with a histone deacetylase inhibitor, and this resulted in increased viral replication. This correlated with changes posttranslational modifications of histones at viral promoters. Thus, the development of this viral replication system for HBV genotype F will facilitate studies on the regulation of viral replication and the identification of new antiviral drugs.

Introduction

Hepatitis B virus (HBV) is a small, enveloped DNA virus and the prototypic member of the family *Hepadnaviridae*. The HBV genome is a partially double-stranded circular DNA molecule of 3.2 kb. Following entry, capsid uncoating, and nuclear transport, host DNA repair enzymes complete the strand synthesis of the incoming genomic DNA to generate a covalently closed circular DNA (cccDNA) molecule that serves as a template for viral transcription [1]. Viral transcription is regulated through four viral promoters, two enhancer elements, and a negative regulator [1–3]. The viral cccDNA is maintained in the infected cell nucleus as episomal DNA and is organized as a minichromosome, forming the typical "beads-on-a-string" structure of cellular chromatin [4–6]. Histones H3 and H4 are associated with the HBV minichromosome and are posttranslationally modified [7, 8]. Consistently, HBV replication correlates temporally with the acetylation of H3 and H4 bound to the minichromosome and with the recruitment of enzymes that acetylate histones, such as p300 and CBP [8, 9]. In contrast, the binding of histone deacetylase 1 (HDAC1), which removes acetyl groups from histone proteins of the HBV minichromosome,

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correlates with histone hypoacetylation and low levels of virus in the blood of HBV-infected patients [8].

The viral cccDNA intermediate is transcribed in the cell nucleus into four major partly overlapping transcripts (3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb in length) that are translocated into the cytoplasm for viral protein translation. The 3.5-kb transcript of the viral genome, named pregenomic RNA (pgRNA), directs synthesis of the polymerase, core and precore viral proteins. Additionally, in the cytoplasm, the pgRNA within immature viral particles serves as the template for reverse transcription to generate the DNA genome, which is incorporated into mature particles [1, 2]. The 2.4-kb and 2.1-kb transcripts generate the envelope proteins including the hepatitis B surface antigen (HBsAg), whereas the 0.7-kb transcript encodes the small viral transactivator X (HBx) protein [10]. The precore protein contains a leader sequence for transportation into the endoplasmic reticulum (ER), where it is processed and secreted as hepatitis B e antigen (HBeAg) [11, 12]. During translation, HBsAg is either inserted into the ER membrane, where it can assemble with the viral capsid to form infectious virions, or secreted into the medium as free HBsAg.

Due to the unusual mechanism of DNA replication, which involves reverse transcription of the pgRNA intermediate by the viral polymerase enzyme, which lacks proofreading activity, the global spread of HBV has led to high sequence heterogeneity among the different circulating viruses. To date, HBV isolates are classified into ten different genotypes (named A-J) and several subtypes that possess distinct geographic distributions worldwide [13, 14]. Broadly, HBV genotypes B and C are prevalent in Asia, whereas genotype A circulates in Africa and genotype D is predominant in India [13]. The HBV genotype G has a worldwide distribution, and genotype H circulates mainly in Central America [15, 16]. The HBV genotypes I and J have been found recently in Asia and Japan, respectively [17, 18]. Finally, HBV genotypes E and F are present in Africa and Central and South America, respectively [19]. Generally, the genomic diversity, pathological features, and antiviral treatment outcomes [20, 21] of the European, North American, and Asian HBV genotypes (A to D) [22, 23] have received greater attention and pharmacological efforts than those of viruses circulating in other areas, such as genotypes E to H [24, 25]. Similarly, clinical and virological properties of HBV genotypes have been only precisely studied for HBV genotypes A to D. In general, genotype B is associated with milder liver diseases than genotype C, and patients with genotype A infections have a better prognosis than those with genotype D [26]. Other HBV genotypes, including genotype F, have not been studied thoroughly at the clinical or molecular level [27].

In previous studies using restriction fragment length polymorphism analysis, the HBV genotype F was found to be the prevalent in Chile (84 %) [28]. Phylogenetic analysis of complete genome sequences from crude amplified viral DNA derived from Chilean chronic patients showed that they were infected with HBV genotype F1b [29]. Furthermore, we have recently described the cloning and full DNA sequence of an HBV strain recovered from samples from a patient who was chronically infected with HBV subgenotype F1b [30]. Here, we describe the replication competence and functionality of this molecular HBV genotype F1b clone, which we have named HBV clone 4.5.

Materials and methods

Cells and cell culture

Huh-7 and HepG2 human hepatocarcinoma cell lines were cultured in DMEM containing 10 % fetal calf serum (Gibco, Life Technologies) under 5 % CO_2 at 37 °C. For sodium butyrate (Sigma Aldrich) treatment, of the drug was added at a concentration of 5 mM 16 h before transfection, and cells were maintained upon the addition of 1.3 μg of HBV DNA. The cells were collected for analysis 24 h later.

Transient transfection with HBV DNA

Full-length HBV genomes were obtained from plasmid DNA by restriction enzyme digestion with 10 U of SapI (Thermo Scientific) for 3 h at 37 °C [31]. Digested DNA was then purified using a Wizard SV Gel and PCR Clean-Up System (Promega). Cells were seeded at a density of 6×10^5 cells per well in 60-mm dishes and transiently transfected 24 h later with linear monomeric HBV DNA using Lipofectamine 2000 Reagent (Invitrogen).

Purification of the viral cytoplasmic DNA intermediate and cccDNA

Transfected cells were treated with lysis buffer (0.5 % Nonidet P40; 10 mM Tris-Cl, pH 7.5; 50 mM NaCl; 1 mM EDTA) in the presence of protease inhibitors. The lysate was transferred to a tube, resuspended by inversion, and allowed to stand on ice for 4 min. Nuclei were pelleted by centrifugation at 2,400 × *g* for 10 min. The supernatant was treated with 300 U of DNase I (Winkler, Chile) for 1 h at 37 °C. Proteins were digested with 0.4 U of proteinase K (New England Biolabs) at 37 °C overnight. Nucleic acids were purified by phenol:chloroform (1:1) extraction and ethanol precipitation. The nuclei were treated with nuclei

lysis buffer (100 mM NaOH; 6 % SDS) and vortexed [32], and nuclei were then incubated at 37 °C for 30 min, after which 600 mM sodium acetate was added, the sample was centrifuged for 9,600 × *g* for 20 min) and the pellet was discarded. The cccDNA was purified twice by phenol:chloroform (1:1) extraction and then precipitated with ethanol [32]. Resuspended cccDNA fraction was treated with Plasmid-Safe ATP-Dependent DNase (Epicentre) [8]. qPCR was performed in a real-time PCR Cycler Rotor Gene Q (QIAGEN) using Kapabiosystems Master Mix (2x) Universal. The following primers were used to analyze the cccDNA: Fw, 5′-ACT CTT GGA CTT TCA GGA AGG-3′; Rev, 5′-TCT TTA TAA GGG TCA ATG TCC AT-3′. The following qPCR primers, corresponding to the core promoter region of HBV, were used to analyze cytoplasmic intermediates: Fw, 5′-GGA AGG TCA ATG ACC TGG ATC-3′; Rev, 5′-ATG CCT ACA GCC TCC TAA TAC-3′.

Viral cccDNA chromatin immunoprecipitation (ChIP) assays

Cells were transfected with 11.7 μg of linear HBV DNA in 150-cm dishes at a density of 5.4 × 10^6 cells per well and were treated with sodium butyrate (Sigma Aldrich). Cells were collected 24 h after transfection and were incubated for 10 min with 1 % formaldehyde with gentle agitation at room temperature. The reaction was quenched with 125 mM glycine. Cells were washed twice with 1x PBS and resuspended in 250 μL of cell lysis buffer (5 mM HEPES, pH 8.0; 85 mM KCl; Triton X-100 and proteinase inhibitors) and homogenized 10 times using a loose pestle. The cell extract was collected by centrifugation at 5400 × *g* for 1 min at 4 °C, resuspended in 250 μL of nuclei buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1 % SDS and protease inhibitors), and allowed to stand for 10 min on ice. Then, 250 μL of IP dilution buffer (200 mM Tris-HCl, pH 8.0; 20 mM EDTA; 500 mM NaCl; 10 % Triton X-100; 1 % SDS and protease inhibitors) was added. Chromatin was sheared in a Bioruptor water bath sonicator (Diagenode Inc.) to obtain fragments up to 400 bp. Extracts were sonicated at high power for four pulses of 10 min each (30 s on, 30 s off) and centrifuged twice at 16,000 × *g* for 10 min at 4 °C. The supernatant was collected and pre-cleared by incubation with 2 μg of normal IgG and 20 μL of protein A (Merck, Millipore) for 2 h at 4 °C, with rotation. The chromatin was centrifuged at 4000 × *g* for 2 min at 4 °C, after which the supernatant was collected and immunoprecipitated with specific antibodies (see below) for 12-16 h at 4 °C. The immunocomplexes were recovered by the addition of 20 μL of protein A agarose beads, followed by 1 h of incubation with rotation at 4 °C. Immunoprecipitated complexes were washed once with sonication buffer (50 mM HEPES, pH 7.9; 140 mM NaCl; 1 mM EDTA, pH 8.0; 1 % Triton X-100; 0.1 % sodium deoxycholate; 1 % SDS), twice with LiCl buffer (500 mM LiCl; 100 mM Tris-HCl, pH 8.0; 1 % Nonidet P40; 0.1 % sodium deoxycholate), and once with Tris-EDTA buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0), each time for 5 min at 4 °C, followed by centrifugation at 4000 × *g* for 5 min. The protein-DNA complexes were eluted by incubation with 100 μL of elution buffer (50 mM $NaHCO_3$ and 1 % SDS) for 15 min at 65 °C. Extracts were centrifuged at 16,000 × *g* for 1 min, the supernatant was collected, and 200 mM NaCl was added. To reverse the cross-linking, chromatin was incubated for 12-16 h at 65 °C. At the same time, 10 μg of RNase A (Invitrogen) was added. Proteins were digested with 100 μg of proteinase K per mL for 2 h at 50 °C, and the DNA was recovered using a DNA Clean & Concentrator (Zymo Research). The following qPCR primers were used to analyze the core promoter region: Fw, 5′-GGA AGG TCA ATG ACC TGG ATC-3′; Rev, 5′-ATG CCT ACA GCC TCC TAA TAC-3′. The following qPCR primers were used to analyze the X promoter region: Fw, 5′-ATT GGC CAT CAG CGC ATG CG-3′; Rev, 5′-AGC TGC AAG GAG TTC CGC AGT-3′. The following qPCR primers were used to analyze the PreS1 promoter region: Fw, 5′-CCC TAT TAT CCT GAT AAC GTG G-3′; Rev, 5′-GCT ACG TGT GGA TTC TCT CTT-3′. The following antibodies were used for ChIP assays: anti-acetylated-H3 (06-599, Merck, Millipore) and anti-total H3 (1791, Abcam). Data were processed as follows: from the Ct value obtained from the qPCR, we quantified immunoprecipitated viral DNA with respect to a standard curve prepared with the HBV genome. We eliminated the background value obtained with the IgG control and then normalized the values to GAPDH as a loading control, and to cccDNA to reflect equal viral DNA loading. For H3ac, we normalized the data against immunoprecipitated H3.

Immunofluorescence

Huh-7 cells were transfected with HBV DNA and at 24 h post-transfection were fixed with 2 % formaldehyde for 20 min and washed three times with 1x PBS. Then, 100 mM glycine was added, and the cells were washed with 1x PBS. Cells were permeabilized with 0.5 % Triton X-100 for 15 min and washed twice with 1x PBS. Cells were blocked with 3 % BSA in 1x PBS and incubated overnight at 4 °C with anti-Core polyclonal antibody (B0586, Dako). Cells were rinsed three times with 1X PBS, incubated for 1 h with the secondary antibody conjugated with Alexa 488 (Rockland), and washed with 1x PBS. The cells were incubated with 1x DAPI (Sigma-Aldrich) for 5 min and washed again with 1x PBS. To analyze immunofluorescence data, samples were imaged using an Olympus BX51 microscope.

Electron microscopy

Huh-7 cells were transfected with purified HBV clone 4.5 DNA and then processed for electron microscopy. Transfected cells were harvested, washed with phosphate-buffered saline, and fixed in 4 % paraformaldehyde and 1 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Cells were then washed in phosphate buffer, harvested, and postfixed with 1 % osmium tetroxide. After dehydration in acetone, cell pellets were embedded in Epon resin that was allowed to polymerize for 24 h at 60 °C. Ultrathin sections were then made with an ultramicrotome, collected on copper grids, and stained with 1 % uranyl acetate and 1 % lead citrate. For immuno-EM, ultrathin sections were incubated with the primary anti-core polyclonal antibody, and then with goat anti-rabbit secondary antibody conjugated with 5-nm gold particles before staining. Grids were then observed with a Philips Tecnai 12 Biotwin transmission electron microscope.

Statistical analysis

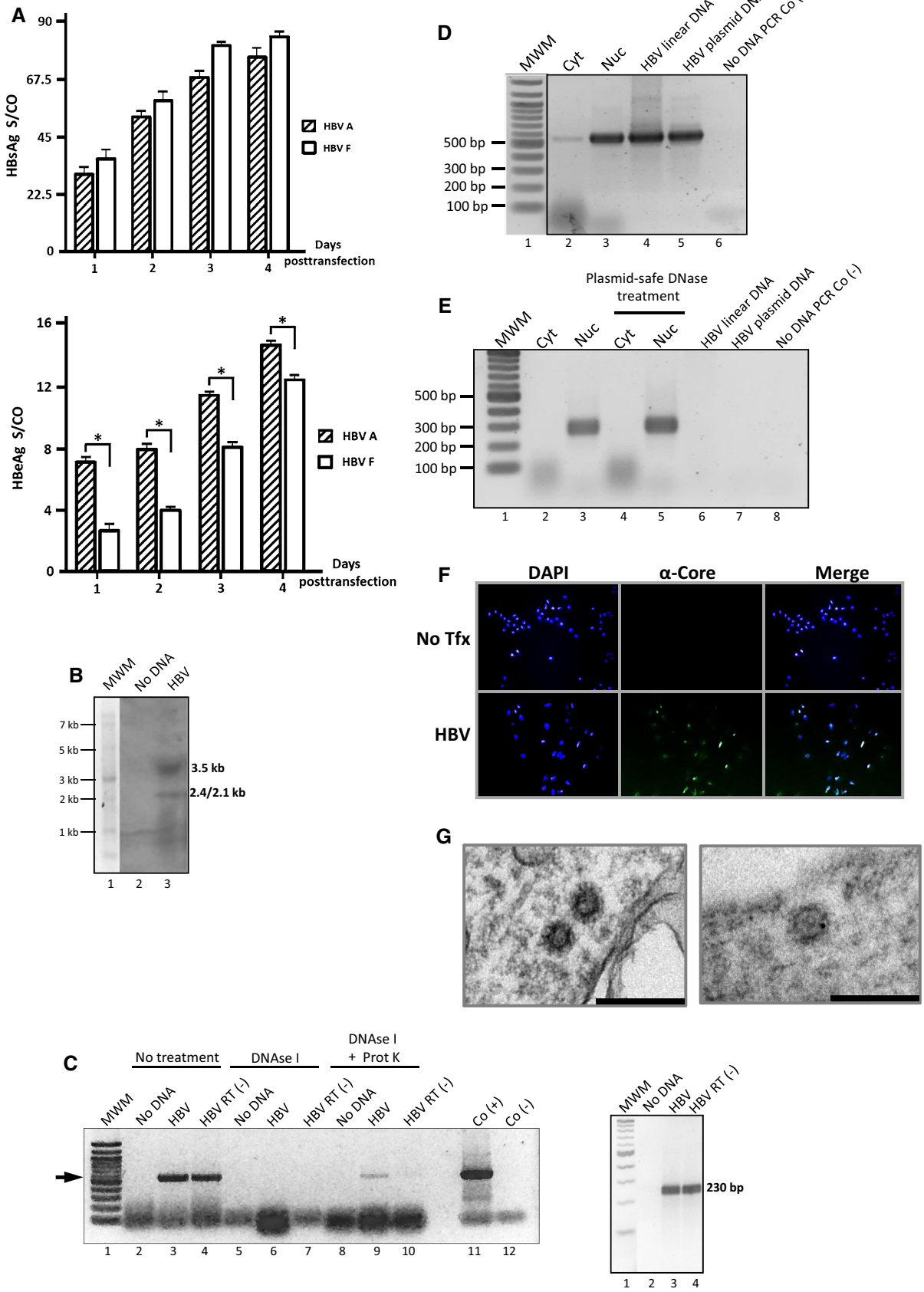
The data presented from at least three separate experiments were expressed as the mean ± SD. The data were evaluated by Student's *t*-test or one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

Replication of HBV clone 4.5 in human hepatocarcinoma cells

We have recently described the cloning and full DNA sequence of HBV clone 4.5, corresponding to HBV subgenotype F1b [29, 30], and the aim of this report was to study its replication in cultured cells. Plasmid DNA was digested with *Sap*I restriction enzyme, and the released viral DNA, which lacks any exogenous sequence, was purified, quantified, and used for transfection of human hepatoma cells. We investigated the time course of secretion for both HBsAg and HBeAg viral antigens into the supernatant of transfected cells. These two viral secretory gene products are normally used as markers of gene expression, whereas HBeAg is also considered a marker of viral replication [33–35]. For comparison, we also tested another functional DNA clone available in our laboratory, corresponding to HBV genotype A, one of the most common HBV genotypes used in research, which was obtained by similar procedures as for HBV clone 4.5 [28, 31] (data not shown). Huh-7 cells were transfected with HBV DNA, and supernatant samples were collected daily and analyzed using the ARCHITECT system (Abbot). The upper and lower panels of Figure 1A show the time course of secretion of HBsAg and HBeAg, respectively, into the supernatant for both viral constructs. Both viral clones displayed similar sustained and increased secretion of both viral antigens over time, and at comparable levels. Whereas the detection of HBsAg indicates its correct expression and secretion into the supernatant, the detection of HBeAg reflects functionality of the transcription from the viral precore promoter for both viral constructs. However, over time, HBV clone 4.5 secreted significantly less ($P < 0.05$) HBeAg than did the HBV genotype A clone (Fig. 1A, lower panel). Additionally, DNase-treated supernatant

Fig. 1 Replication of HBV clone 4.5 in human hepatocarcinoma cells. (A) Kinetics of secretion of HBsAg (upper panel) and HBeAg (bottom panel) from cells transfected with HBV clone 4.5 (genotype F) and an available clone of HBV genotype A. Huh-7 cells were transfected with viral DNA, and supernatants were collected at the indicated time points. Supernatants were analyzed using an ARCHITECT i1000 analyzer (Abbott). In each case, data are presented as a signal-to-cutoff (S/CO) ratio. Results are expressed as mean ± SD. Statistically significant changes ($P < 0.05$) are indicated by asterisks above the brackets. (B) Detection of viral RNA isolated from cells transfected with HBV clone 4.5. Huh-7 cells were transfected with DNA from HBV clone 4.5, and after 48 h, total RNA was isolated from the cells. Ten μg of total RNA was transferred to a nylon membrane and hybridized with a [^{32}P]-labeled full-length HBV 4.5 probe. (C) HBV DNA replication intermediates from cells transfected with HBV clone 4.5. Huh-7 cells were transfected with either HBV clone 4.5 or HBV clone 4.5 RT(-) and were processed 24 h post-transfection. The left panel shows conventional PCR detection of cytoplasmic HBV DNA replication intermediates. Cytoplasmic samples were treated as indicated: no treatment (lanes 2-4), DNase I treatment (lanes 5-7), DNase I and proteinase K treatment (lanes 8-10). The right panel shows conventional PCR detection of nuclear cccDNA. (D) Conventional PCR reactions were performed to detect the core coding region in samples of both cytoplasmic and nuclear viral DNA isolated from cells transfected with HBV clone 4.5. Samples of cytoplasmic viral DNA (lane 2), nuclear viral DNA (lane 3), and HBV linear monomer DNA (lane 4), HBV plasmid DNA (lane 5) are indicated. Lane 6, no-DNA PCR control reaction. (E) Conventional PCR reactions carried out to detect an approximately 230-base-pair region covering the junction region of the circularized viral cccDNA in samples of both cytoplasmic and nuclear viral DNA isolated from cells transfected with HBV clone 4.5. Samples of cytoplasmic viral DNA (lane 2) and nuclear viral DNA (lane 3) without Plasmid-Safe DNase treatment, HBV linear monomer DNA (lane 6), and HBV plasmid DNA (lane 7) are indicated. Lanes 4 and 5, samples of cytoplasmic and nuclear viral DNA, respectively, with Plasmid-Safe DNase treatment; lane 8, represents no-DNA PCR control reaction. (F) Indirect immunofluorescence detection of the HBV core protein. Huh-7 cells were transfected with purified HBV clone 4.5 DNA, and cells were then processed for indirect immunofluorescence, using an HBV anti-core polyclonal antibody and a secondary antibody conjugated with Alexa 488. (G) Electron microscopy analysis of transfected cells. Huh-7 cells were transfected with purified HBV clone 4.5 DNA, and cells were then processed for electron microscopy. Left, intracytoplasmic HBV particles; right samples treated with an HBV anti-core polyclonal antibody and then with a secondary antibody conjugated to 5-nm gold particles. Bars indicate 100 nm


A
HBsAg S/CO
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67.5
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HBV A
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1 2 3 4
HBeAg S/CO
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HBV linear DNA
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samples from day 4 were used to determine the viral load of both constructs, utilizing the COBAS TaqMan HBV Test (Roche). Consistent with the replication results obtained by measuring HBeAg secretion, samples of HBV genotype A gave a mean viral load of 2.1×10^6 copies/mL, whereas samples of HBV clone 4.5 gave a mean viral load of 6.3×10^4 copies/mL, reflecting that HBV clone 4.5 actively replicates, although at a lower level than the genotype A clone.

We next wanted to detect viral replication intermediates from Huh-7 cells that were transiently transfected with HBV clone 4.5. Total RNA was isolated 48h post-transfection, transferred to nylon membranes, and hybridized with a [^{32}P]-labeled full HBV DNA genomic probe. The RNA bands for the most abundant viral transcripts, such as pregenomic RNA (3.5 kb), and those corresponding to preS1/S transcripts (2.4/2.1 kb) were clearly detected (Fig. 1B, lane 3). This indicates that HBV clone 4.5 is transcriptionally active when transiently expressed in hepatoma cells and that the viral transcripts generated are of the expected size. Total RNA isolated from control non-transfected Huh-7 cells (lane 2) did not detectably cross-hybridize with the viral probe.

To further analyze HBV DNA replication intermediates from HBV clone 4.5, we constructed a defective HBV mutant bearing a point mutation (YMDD to YMHD) within the active-site motif of the reverse transcriptase [36–38]. This clone was constructed by site-directed mutagenesis within the HBV clone 4.5 backbone and was called HBV RT(-). Huh-7 cells were transiently transfected with each construct, and after 24 h, both cytosolic and nuclear fractions were obtained as described previously [8]. Equivalent amounts of the cytosolic fraction were either saved, treated with DNase I, or treated with DNase I and then with proteinase K before subjecting them to phenol chloroform extraction and ethanol precipitation [8]. Viral HBV DNA was then detected by conventional PCR targeting the core protein coding region. As shown in the left panel of Figure 1C, for equivalent amounts of transfected DNA from these two viruses (lanes 3 and 4), only DNA after DNase I (lanes 6 and 7) and subsequent proteinase K treatment was significantly detected by conventional PCR from cells transfected with HBV clone 4.5 (lane 9), indicating that the detected DNA is specifically associated with and protected by cytoplasmic HBV particles. The very low level of viral DNA that is associated with intracytoplasmic particles detected by PCR indicates that the HBV RT(-) construct is defective (lane 10) [36–38]. Nuclear fractions were subjected to low-molecular-weight DNA isolation as described previously [8, 32], and purified viral DNA was then subjected to PCR that specifically targeted an approximately 230-base-pair region covering the junction region in the circularized viral cccDNA. After 24 h of transfection, both viral DNA constructs were able to circularize the transfected DNA, and the specific conventional PCR results probably reflect the presence of significant amounts of transfected and circularized DNA (Fig. 1C, right panel, lanes 3 and 4).

We next wanted to test and verify the specificity of our PCR reactions for detection of predominantly viral cccDNA and not the other viral DNA molecules also present in the fractionated samples. For this, we tested samples of viral DNA isolated from either the cytoplasm or the nucleus of cells transfected with linear monomers of clone HBV 4.5 as described previously. First, we used conventional PCR with primers that targeted the core coding region, as before. As shown in Fig. 1D, and excluding the control (lane 6), PCR reactions resulted in DNA amplification from all of the samples, although at different levels, including cytoplasmic and nuclear HBV DNA (lanes 2-3), monomeric HBV DNA (lane 4), and HBV plasmid DNA (lane 5), since the target core sequence is present in all of these viral DNAs. We then tested the specificity of our conventional PCR for detection of cccDNA. As mentioned above, this PCR specifically targeted an approximately 230-base-pair region covering the junction region in the circularized viral cccDNA. Additionally, we treated the samples with Plasmid-Safe DNase, which should degrade linear and relaxed circular HBV DNAs and thus prevent their presence in the nuclear DNA fraction. As shown in Fig. 1E, only samples from nuclear HBV DNA gave amplification of the viral cccDNA (lanes 3 and 5), where the treatment of samples with Plasmid-Safe DNase seemed to improve the product amplification. Samples of either cytoplasmic (lane 2 and 4), linear monomeric (lane 6) or plasmid (lane 7) HBV DNAs gave no amplification products. Together, these results confirm the specificity of this assay for the detection of viral cccDNA and provide clear evidence that cccDNA is formed after transfection in our system.

We then aimed to detect viral proteins from cells transfected with HBV clone 4.5. For this, Huh-7 cells were transfected with the purified viral DNA, and the cells were processed for detection of the HBV core protein by indirect immunofluorescence. The HBV core protein was only detected in cells transfected with the viral DNA (Fig. 1D), and the staining pattern indicated that the HBV core protein is localized in both the cytoplasmic and nuclear compartments, probably due to nucleocytoplasmic shuttling [39]. Transfected cells were also processed for electron microscopy analysis. Intracytoplasmic HBV particles were detected in transfected Huh-7 cells (Fig. 1E, left panel), and their appearance was consistent with an enveloped and icosahedral particle of approximately 45 nm in diameter. We also specifically detected intracytoplasmic HBV particles via immunogold labeling after incubation with a

HBV anti-core antibody (Fig. 1E, right panel). We were unable to identify these viral assemblies from non-transfected Huh-7 cell samples, processed in parallel in each case.

The HBV clone 4.5 HBx-deficient mutant displays impaired replication in HepG2 cells but not in Huh-7 cells

It has been proposed previously that HBV replication shows different requirements in distinct cell types, probably due to the presence or absence of distinct sets of regulatory cell factors [10]. For example, a mutant HBV genome bearing a mutation introducing a premature stop codon within the viral HBx gene led to decreased levels of HBV replication intermediates in HepG2 cells but not in Huh-7 cells [40–42]. To test HBV clone 4.5, we transfected either Huh-7 or HepG2 cells and then compared the secretion of HBV antigens into the supernatant from the parental HBV clone 4.5 with that of an HBV mutant defective in the expression of the HBx protein, which we named HBV 4.5 X(-) [41]. The HBV X(-) mutant possess a premature stop codon in the HBx gene and thus does not express the HBx protein (data not shown). We first compared the levels of gene expression in Huh-7 cells by measuring HBsAg, and we compared the levels of replication by measuring HBeAg [33–35]. For this, Huh-7 cells were transfected with each purified viral DNA, and supernatant samples were collected daily and analyzed using the ARCHITECT system (Abbott) for detection of both viral antigens. The kinetics of HBsAg and HBeAg secretion from Huh-7 cells for both viral DNA constructs showed that secretion of HBsAg and HBeAg into the supernatant was sustained at comparable levels, and thus, these two viral constructs probably replicate at equivalent levels in Huh-7 cells (Fig. 2A, left and right panels).

We then compared the secretion of viral antigens after transfection of HepG2 cells. HepG2 cells were transfected with each purified viral DNA as before, and supernatant samples were collected daily and analyzed using the ARCHITECT system (Abbott) for detection of both HBsAg and HBeAg viral antigens. HBV clone 4.5 displayed sustained secretion of HBsAg into the supernatant, albeit at a lower level than that obtained with transfected Huh-7 cells (Fig. 2B, left panel). This difference in the total viral antigen secretion level between Huh-7 and HepG2 cells probably reflects the higher transfection efficiency of Huh-7 cells. HBsAg secretion from HepG2 cells transfected with HBV 4.5 X(-) DNA was significantly lower ($P < 0.05$) than that of wild-type HBV 4.5 clone, starting from day 3 posttransfection. The secretion of HBeAg from HepG2-transfected cells is shown in Figure 2B, right panel. Although HBV 4.5 showed continuous and sustained secretion of this antigen, the secretion of HBeAg from HBV X(-)-transfected cells was significantly reduced ($P < 0.05$), starting from day 3 posttransfection, which probably also reflected an impaired replication step with this construct in this cell line.

Sodium butyrate activates the HBV cccDNA chromatin state

The HBV cccDNA is maintained within the hepatocyte nucleus as a minichromosome is and then subjected to regulation mediated by the minichromosome structure [43]. Therefore, we next investigated the functionality of the HBV-derived cccDNA from an *in vitro* HBV replication and transcription system. Because the acetylation of histones H3 and H4 that are bound to the HBV cccDNA stimulates HBV replication [8, 9], we examined whether a well-known class I/II histone deacetylase inhibitor such as sodium butyrate [44] could stimulate HBV replication in this culture system. We incubated Huh-7 cells with sodium butyrate before and during HBV DNA transfection with purified viral DNAs (Fig. 3A). To compare two different HBV genomes, we transfected cells with the HBV genomes of genotypes F and A. We first investigated the effect of the compound on viral DNA replication intermediates. When transfected with either HBV genotype F or A (Fig. 3B, left), the levels of cytoplasmic DNA intermediates significantly increased after sodium butyrate treatment compared to those in untreated cells. The amount of cccDNA significantly increased upon sodium butyrate treatment only in cells transfected with HBV genotype F (Figure 3B, right). We next examined the acetylation state of histone H3 that was bound to HBV cccDNA after sodium butyrate treatment. For this, we performed chromatin immunoprecipitation (ChIP) assays, by immunoprecipitating total histone H3 and acetylated histone H3 and performing quantitative PCR for three of the HBV promoters. As expected, the results showed that histones H3 were bound to the three viral promoters on the HBV cccDNA of genotypes F and A, confirming that the cccDNA was assembled into chromatin in our culture system (Fig. 3C). Notably, we observed that the level of histone H3 after sodium butyrate treatment was significantly lower at three viral promoters of HBV genotype F (Fig. 3C, left) and two viral promoters of HBV genotype A (Fig. 3C, right). We then investigated the acetylation state of the HBV cccDNA-bound histone H3, and ChIP-qPCR analysis indicated that the histone H3 at the core, HBx and PreS1 promoters of genotype F became hyperacetylated upon sodium butyrate treatment (Fig. 3D, left). When analyzing viral promoters of genotype A, we observed that the core and PreS1 promoters became hyperacetylated upon sodium butyrate treatment (Fig. 3D, right). Thus,

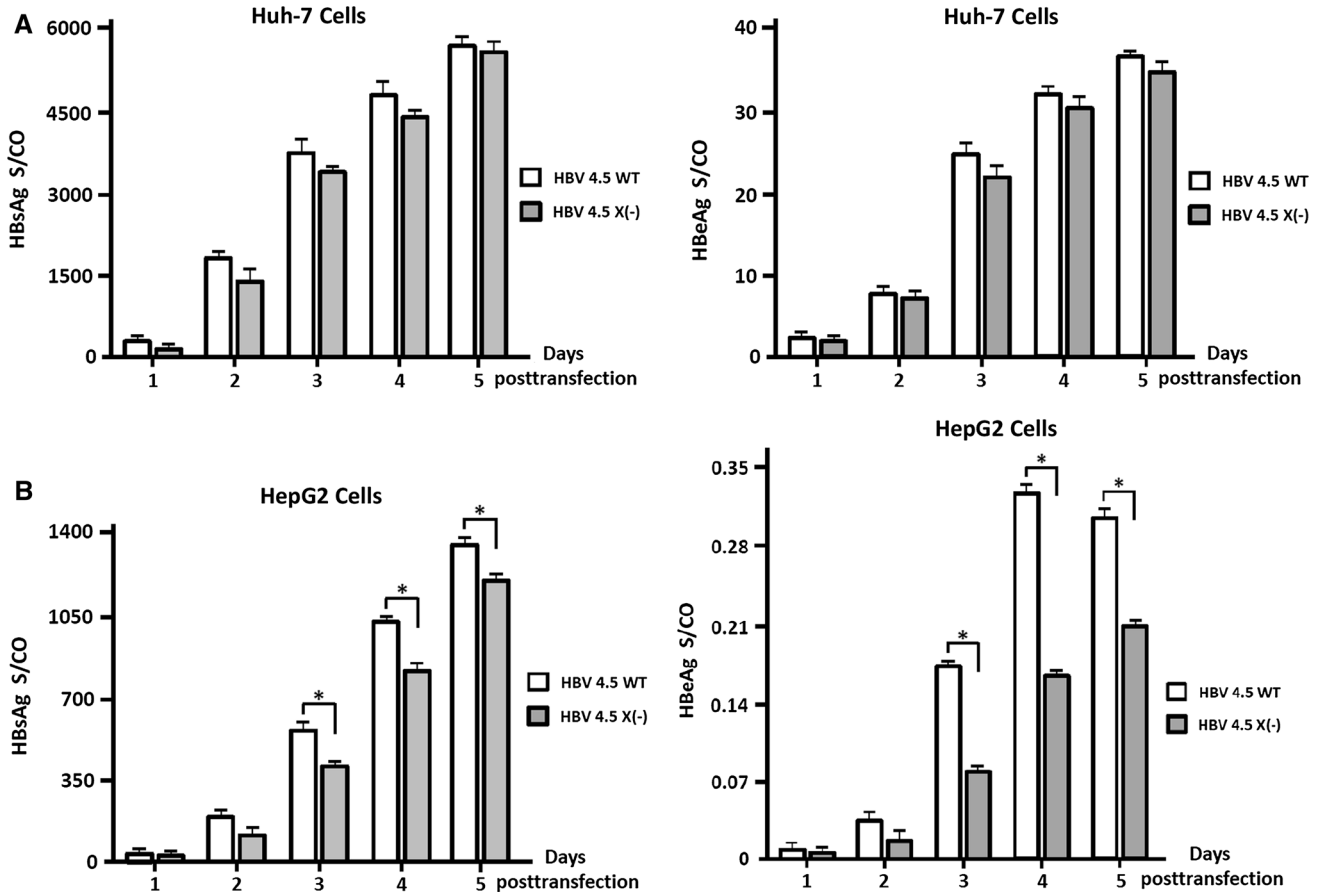

Fig. 2 Kinetics of the secretion of HBsAg and HBeAg in two different hepatocarcinoma cell lines transfected with HBV clone 4.5. (A) Huh-7 and (B) HepG2 cells were transfected with the parental HBV clone 4.5 DNA or with its mutant HBV X(-). Supernatants were collected at different time points as indicated, and samples were analyzed using an ARCHITECT i1000 analyzer (Abbott) to detect either HBsAg (left panel) or HBeAg (right panel). In each case, data are presented as a signal-to-cutoff (S/CO) ratio. Results are expressed as mean ± SD. Statistically significant changes ($P < 0.05$) are indicated with asterisks above the brackets

after sodium butyrate treatment, viral promoters have, on the one hand, reduced levels of histone H3, and, on the other hand, hyperacetylated histone H3 compared to cccDNA derived from untreated cells. These observations correlated with the observed higher levels of viral DNA cytoplasmic intermediates after sodium butyrate treatment, suggesting that HBV cccDNA chromatin is dynamic and that sodium butyrate can convert the HBV cccDNA chromatin to an activated state.

Discussion

In this study, we analyzed the replication competence of a new HBV molecular clone. HBV clone 4.5 was obtained from serum samples of a Chilean chronically infected patient [30] via a widely used procedure [31]. Phylogenetic sequence analysis indicated that HBV clone 4.5 corresponded to the viral subgenotype F1b [28, 29]. Upon transfection of human hepatoma cell lines, HBV clone 4.5 induced sustained secretion of HBsAg, which is a marker for viral gene expression [34]. Additionally, HBeAg, a marker for viral replication, was secreted at high levels, indicating active viral replication in this culture system [33], and at a level similar to that of another clone from genotype A, one of the most commonly used HBV genotypes in research. Transfected hepatocarcinoma cells were also analyzed for viral intermediates, and we directly detected viral RNA transcripts, which specifically represented both cytoplasmic and nuclear viral DNA in replication. Notably, electron microscopy analyses of Huh-7 cells with ongoing HBV replication allowed us to specifically identify intracytoplasmic viral particles, indicating that HBV clone 4.5 can also direct viral assembly. Consistent with our previous data, supernatants from Huh-7 cells transfected with HBV clone 4.5 produced a mean viral load of 6.3×10^4 copies/mL, indicating that viral particles were released from these cells.

It has been reported previously that HBV is capable of HBx-independent replication in Huh-7 cells, whereas HBV

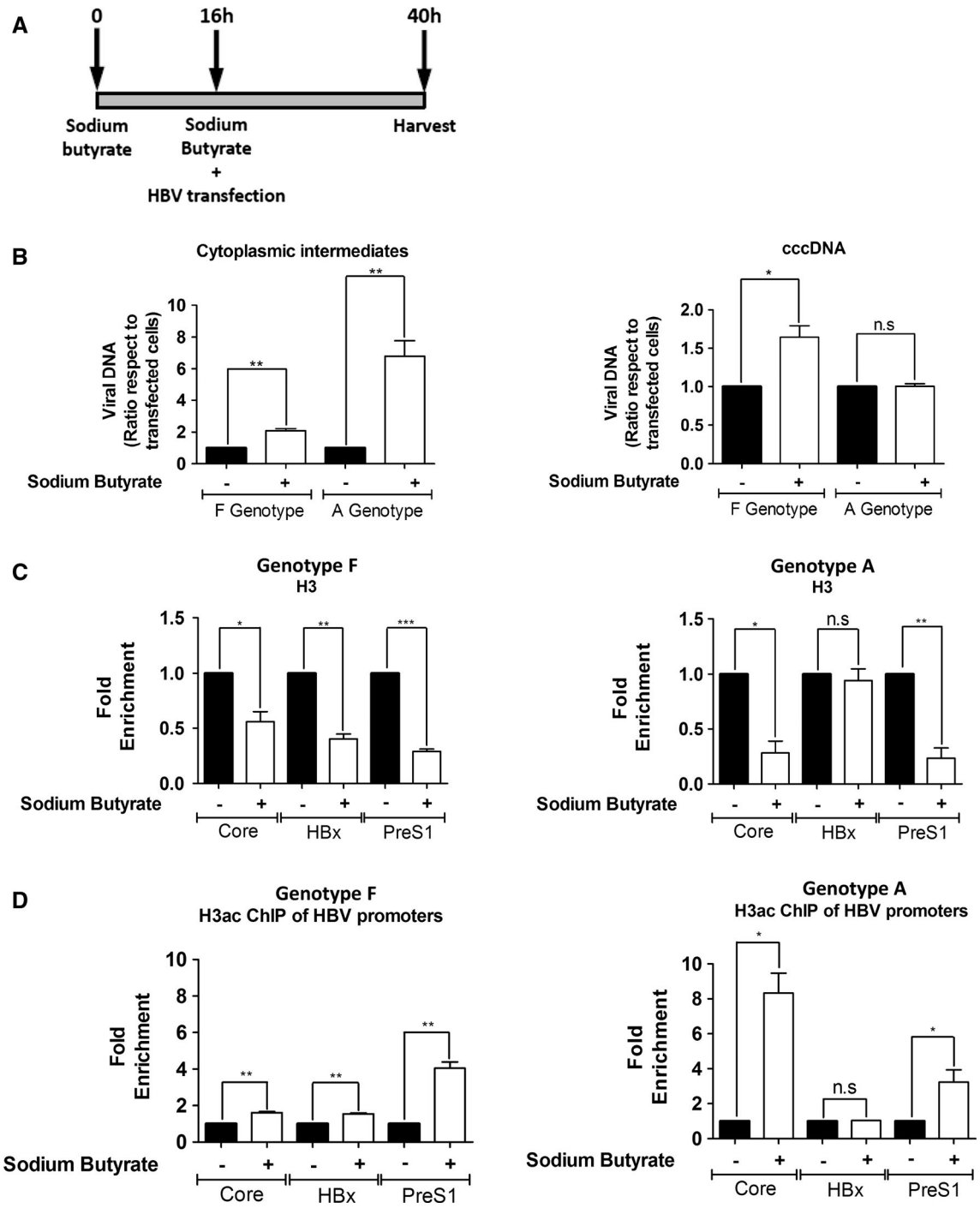

Fig. 3 Activation of the HBV cccDNA chromatin state by treatment with sodium butyrate. (A) Schematic illustration of the sodium butyrate treatment of Huh-7 cells transfected with HBV clone 4.5. (B) Cytoplasmic DNA intermediates (left) and viral cccDNA (right) were quantified from HBV-transfected Huh-7 cells that were treated with sodium butyrate or left untreated. The standard deviation was obtained from three independent experiments. (C-D) Chromatin immunoprecipitation (ChIP) assays on total histone H3 (C) and acetylated histone H3 (H3ac) (D) from HBV cccDNA. ChIP analysis was performed on sodium-butyrate-treated or untreated Huh7 cells transfected with the linear HBV genotype F (left) or A (right) clones. DNA fragments bound to histone H3 were immunoprecipitated using antibodies against histone H3, H3ac, or rabbit isotype-matched immunoglobulin G, as a control. Precipitated DNA was quantified by real-time PCR with primers specific for the core, HBx, and PreS1 viral promoters, and the PCR products were normalized as described in Materials and methods. The standard deviation was obtained from three PCR reactions and three independent experiments

replication is HBx-dependent in HepG2 cells [40–42]. In this study, the replication patterns of parental HBV clone 4.5 and the HBV X-mutant were different in Huh-7 and HepG2 cells, as monitored by secretion of both HBsAg and HBeAg viral antigens into the supernatant. Consistent with previous reports, Huh-7 cells supported HBx-independent replication, whereas HBV replication in HepG2 cells was significantly impaired with the HBV X-mutant. The reason for this differential behavior of HBV in different cell lines is currently unknown. In Huh-7 cells, the synthesis of viral proteins or DNA or RNA replicative intermediates and particle export is not affected [41]; however, in HepG2 cells, HBx has been shown to be critical for genome replication [42], and HBx affects the synthesis of pgRNA and DNA replication intermediates [45]. As the synthesis of pgRNA is required as the first step of HBV replication [2], transcriptional activation by HBx is possibly associated more strongly with replication efficiency in HepG2 cells than in Huh-7 cells, as it has been shown that HBx is recruited to cccDNA [9].

Transiently transfected, inducible, integrated, or baculovirus-based cell-line systems have been used to study many important stages of HBV replication and its particular genomic organization [9, 46–49]. For instance, HepG2.2.15 cells are a well-known HBV-expressing hepatoblastoma cell line that is stably transfected with a vector that contains two head-to-tail dimers of the HBV genome [50]. The HepAD38 cell line replicates HBV under the control of tetracycline [49], and HepG2.2.15 and HepAD38 cells produce viruses corresponding to genotype D and A, respectively. Indeed, most of the described HBV replication systems replicate HBV genomes from genotypes A to D. In contrast, HBV genotypes E and F, which are prevalent in Africa, Central and South America [19] have not undergone extensive molecular, clinical or pharmaceutical research. Accordingly, the replication system of HBV clone 4.5 from genotype F in hepatoma cells presented here furthers research into of one of the less-characterized genotypes of the human hepatitis B viruses.

In the nucleus of infected cells, viral cccDNA is assembled and maintained as a minichromosome by association with nucleosomes [43]. It has been firmly established that the posttranslational modifications present on histone proteins associated with the viral minichromosome regulate either its transcriptional activation or repression. Specifically, ChIP assays of the nuclear viral minichromosome have shown that the acetylation of lysine on histones H3 and H4 correlates with HBV replication, which depends on pgRNA transcription, and on the viremia level in chronically HBV-infected patients [8, 9, 43]. Host histone acetyltransferases, such as CBP, p300, and PCAF/GCN5, and histone deacetylases, such as HDAC1 and hSirt1, are also recruited to the cccDNA [8, 43]. Additionally, the treatment of HBV-transfected cells with class I/II histone deacetylase inhibitors such as valproic acid (VPA) or trichostatin A (TSA) resulted in an increase in cccDNA-bound acetylated H3 and H4 histones, and in an increase in the accumulation of viral cytoplasmic DNA replication intermediates [8, 43].

When we analyzed the functionality of HBV clone 4.5, we observed that, similar to HBV genotype A, HBV clone 4.5 formed cccDNA in the structure of chromatin. The inhibition of class I/II histone deacetylases by treatment with sodium butyrate stimulated viral replication, which correlated with reduced levels of histone H3 on the viral promoters as well as hyperacetylation. Notably, we observed that genotype A replicated more than genotype F and that their viral transcriptional activities correlated with their cccDNA chromatin state. Thus, the HBV clone 4.5 cccDNA replicative intermediate is fully functional. Taken together, our results show that we could generate an active HBV clone representative of genotype F, which mimics the HBV replicative cycle in hepatocarcinoma cell lines. In addition, we observed that we can regulate the viral activity by modulating the cccDNA chromatin state, i.e., the abundance of replicative intermediates. Therefore, the modulation of histone-modifying enzymes that are recruited to the viral chromatin might be a novel target for the development of antiviral drugs for the treatment of chronically infected HBV patients.

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