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Title. Hepatitis B virus basal core promoter mutations show lower replication fitness associated with cccDNA acetylation status

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HIGHLIGHTS

- Basal core promote (BCP) mutations of hepatitis B virus show low replication fitness.
- The precore mutations have no significant effect on viral replication.
- Viral replication capacity of mutants parallels cccDNA acetylation status.
- cccDNA is a target for methylation and is accompanied by DNMT1 upregulation.
- HBV mutants modulate viral replication via cccDNA epigenetic control.

Abstract

In chronic hepatitis B virus (HBV) infection, variants with mutations in the basal core promoter (BCP) and precore region predominate and associate with more severe disease forms. Studies on their effect on viral replication remain controversial. Increasing evidence shows that epigenetic modifications of cccDNA regulate HBV replication and disease outcome. Here we determined the transcription and viral replication efficiency of well-defined BCP and precore mutations and their effect on cccDNA epigenetic control. HBV monomers bearing BCP mutations A1762T/G1764A and A1762T/G1764A/C1766T, and precore mutations G1896A, G1899A and G1896A/G1899A, were transfected into HepG2 cells using a plasmid-free approach. Viral RNA transcripts were detected by Northern blot hybridization and RT PCR, DNA replicative intermediates by Southern blotting and RT PCR, and viral release was measured by ELISA. Acetylation of cccDNA-bound histones was assessed by Chromatin ImmunoPrecipitation (ChIP) assay and methylation of cccDNA by bisulfite sequencing. BCP mutations resulted in low viral release, mRNA transcription and pgRNA/cccDNA ratios that paralleled the acetylation of cccDNA-bound H4 histone and inversely correlated with the HDAC1 recruitment onto cccDNA. Independently of the mutations, cccDNA was a target for methylation, accompanied by the upregulation of DNMT1 expression and DNMT1 recruitment onto cccDNA. Our results suggest that BCP mutations decrease viral replication capacity possibly by modulating the acetylation and deacetylation of cccDNA-bound histones while precore mutations do not have a significant effect on viral replication. These data provide evidence that epigenetic factors contribute to the regulation of HBV viral replication.

Keywords: Hepatitis B virus, cccDNA, epigenetic, viral replication, precore, basal core promoter

1. INTRODUCTION

To-date, hepatitis B virus (HBV) poses a global health problem, with approximately one-third of the world's population having markers of current or past infection (Cassidy et al., 2011). Upon infection of the hepatocyte, the 3.2kb HBV genome is delivered to the nucleus, where the open circular, partially double-stranded DNA (OC DNA) is converted into a covalently closed circular DNA (cccDNA) molecule. The cccDNA exists as a stable non-integrated minichromosome and forms the template for all viral mRNA transcript synthesis (Tuttleman et al., 1986). One of the transcripts termed pre-genomic RNA (pgRNA) is the template for genome replication and its reverse transcription ultimately determines viral replication rate. In addition, it encodes for the core protein (HBcAg) and viral polymerase. In contrast the secreted HBV e antigen (HBeAg) is encoded by the precore transcript (Moolla et al., 2002). The synthesis of the precore protein is directed by the core promoter, which is located between nucleotides (nt) 1591-1822 (Kramvis and Kew, 1999) and also encompasses the basal core promoter (BCP), which is mapped between nt1744-1804 (Buckwold et al., 1996).

During chronic HBV infection (CHB) mutated viral variants are selected, which either abrogate or reduce HBeAg production levels (Huang et al., 2006). One such variant has a double substitution in the BCP region that involves an A to T transversion at nt1762 combined with a G to A transition at nt1764 (A1762T/G1764A) and its appearance is usually followed by the triple A1762T/G1764A/C1766T mutant (Okamoto et al., 1994). These mutations suppress, but do not abolish HBeAg synthesis (Buckwold et al., 1996). The most common mutation that generates an HBeAg-negative phenotype involves a G to A transition at nt1896 (G1896A) of the precore region, often refer to as the precore stop-codon variant and may be accompanied by a second G to A transition at position 1899 (G1899A) (Carman et al., 1989).

Variants with either BCP and precore mutations alone or combined have been associated with more severe forms of liver damage, fulminant hepatitis, reactivation phase of the disease, severe recurrent liver disease following liver transplantation, and the development of hepatocellular carcinoma (HCC) (Baptista et al., 1999;Kao et al., 2003;Munoz et al., 2011;Li et al., 2013). These mutations contribute to HBeAg seroconversion and immune clearance of the WT quasispecies. Studies on the effect of BCP mutations on viral replication remain controversial. Some reports suggest that they increase (Buckwold et al., 1996; Moriyama et al., 1996; Pang et al., 2004), some that they downregulate (Jammeh et al., 2008; Fang et al., 2009; Shi et al., 2012), while others that they do not have any effect on viral replication (Scaglioni et al., 1997; Chun et al., 2000; Yoo et al., 2003). Precore mutated variants have not been associated with differential viral replication capacity (Gunther et al., 1998; Parekh et al., 2003; Jammeh et al., 2008).

Cumulative evidence shows that HBV replication is regulated by epigenetic modifications (Pollicino et al., 2006;Guo et al., 2009;Vivekanandan et al., 2010;Koumbi et al., 2015). In the nuclei of infected hepatocytes, HBV DNA associates with histones combined with HBcAg to form cccDNA minichromosomes (Bock et al., 2001). Chromatin condensation of cccDNA determines its accessibility to regulatory transcription factors and therefore viral gene expression (Bock et al., 2001). The acetylation status of cccDNA-bound histones has been shown to control HBV replication (Pollicino et al., 2006; Belloni et al., 2009; Belloni et al., 2012). The transcriptional control region of cccDNA overlaps with the CpG II island and its

methylation has been shown to impair cccDNA transcription and viral expression (Guo et al., 2009; Vivekanandan et al., 2009; Kim et al., 2011).
[1]

The aim of this study was to investigate the transcription and replication efficiency of well-defined BCP and precore mutations. In contrast to earlier studies on the replication capacity of mutant constructs, we used an established plasmid-free cell system, which recapitulates the full-HBV life cycle after transfection of genome length linear HBV monomers into human hepatoma HepG2 cells (Gunther et al., 1995; Pollicino et al., 2006). The HBV monomers are delivered to the hepatocyte nuclei where they undergo spontaneous circularization and serve as a template for viral transcript synthesis (Gunther et al., 1995; Pollicino et al., 2006). We further aimed to correlate the viral replication capacity of the BCP and precore variants with the acetylation status of cccDNA and the methylation levels of the cccDNA region of the CpG II island, which has not been attempted before in this context.

2. MATERIALS AND METHODS

2.1. Generation of HBV constructs for transient transfection

A replication-competent plasmid (p3.8II) containing a 1.2x (3.8 kb) genome length copy of wild-type (WT) HBV subtype *adr* (genotype C) in a pBluescript II KS (+) background (Stratagene, Ca, USA) served as a template for the preparation of HBV constructs carrying the BCP (A1762T/G1764A and A1762T/G1764A/C1766T) and precore mutations (G1896A, G1899A and G1896A/ G1899A). To generate the mutated genomes, the QuikChange Multisite-directed mutagenesis kit from Stratagene was used, as described in the manufacturer's instructions and using the primers shown in Table 1. The WT HBV-p3.8II template plasmid was kindly provided by Professor Yuan Wang, Academia Sinica, Shanghai, China (Fu and Cheng, 1998). A mutant HBV genome carrying a deletion of 61aa (Δ47-108aa) in the preS1 region was used as an additional internal control. Its construction and failure to express the preS1 transcript has been previously described (Pollicino et al., 2012).

Full-length HBV genomes were amplified from WT or mutant HBV genomes according to the method described by Günther (Gunther et al., 1995), using the P1 sense (HBV positions: nt1821-1841) and P2 antisense primers (HBV positions: nt1823-1806) modified to contain the *HindIII/SapI* sites and the *SacI/SapI* sites, respectively (Table 1). Polymerase chain reactions (PCR) were performed using the FastStart High Fidelity PCR System (Roche Applied Science, Germany) according to the manufacturer's instructions. Each of the 3.2kb amplified HBV fragments were purified from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific, Life Science Research, Lutterworth, UK) and were cloned into a pCRII TA-vector (Invitrogen, Pasley, UK). HBV-pCRII plasmids were then digested with *SapI* (New England Biolabs, Beverly, MA) to release the 3.2kb linear HBV DNA monomers, which were gel purified. Plasmids expressing the HBx or HBeAg proteins were generated by amplifying the relevant coding region from the WT HBV-pCRII construct with the appropriate HBeAg- and HBx- primers modified to contain *EcoRI* and *XbaI* restriction sites (Table 1), and cloning the amplicons into pcDNA3.1(+) following double digestion of both,

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Abbreviations: BCP, basal core promoter; CHB, chronic HBV; cccDNA, covalently closed circular DNA; DNMT, DNA methyltransferases; DS DNA, double stranded DNA; HATs, histone acetyltransferases; HDAC1, class 1 histone deacetylase; OC DNA, open circular DNA; ORFs, open reading frames; pgRNA, pre-genomic RNA; SS DNA, single stranded DNA; TSA, Trichostatin.

insert and vector, with *EcoRI* and *XbaI* (New England Biolabs, Beverly, MA). All constructs were sequenced to confirm the presence of the mutations and to ensure that no additional mutations were introduced in the process.

2.2. Transient Transfection experiments

Linear HBV monomers were used for transient transfection of HepG2 cells by lipofection. Briefly, HepG2 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% foetal bovine serum, 2mM L-glutamine, 100IU penicillin ml^{-1} and 100μg streptomycin ml^{-1}. $4x10^5$ cells were seeded in 60mm diameter dishes (Corning, Flintshire, UK) and the day after 500ng of *SapI* digested HBV monomers complexed with Lipofectamine Plus (Invitrogen) were added. Culture medium was changed 24h post-transfection, and cells were harvested 24h later (total 48h). When indicated, the HBeAg-pcDNA3.1 or HBx-pcDNA3.1 plasmids were co-transfected with the BCP double and triple mutated monomers to deliver 20ng or 100ng of the HBeAg and HBx constructs to the cells, respectively. The histone deacetylase (HDAC) inhibitor, Trichostatin A (TSA) (Sigma-Aldrich, St Louis, MO) (300nM), or the DNMT inhibitor, Curcumin (Sigma-Aldrich) (50μM) were added 24h post transfection. The doses of the epigenetic inhibitors were optimised at 48h post-transfection, which is the peak of HBV DNA replication in the present plasmid-free transfection system (Pollicino et al., 2006). All transfections included triplicate controls of 1μg of reporter plasmid expressing a green fluorescent protein (GFP) to monitor transfection efficiency by fluorescence-activated cell sorting (FACS) analysis and ranged between 43-50%.

2.3. RNA analysis

Total RNA was extracted from HepG2 cells 48h after transfection with 500ng of *SapI* digested HBV DNA, using Trizol reagent (Invitrogen) as recommended by the manufacturer. RNA samples were treated with RNase-Free DNase (Qiagen, Crawley, UK) for 10min at 25^oC and then purified using the RNeasy mini kit (Qiagen). RNA integrity was confirmed by agarose gel electrophoresis under UV and RNA quality and quantity was measured using an ND-1000 spectrophotometer (NanoDrop Technologies) at 260nm.

2.3.1. Northern Blot Analysis

For Northern blot analysis, 10μg of total RNA per sample was separated on a 1% formaldehyde-agarose gel and blotted onto a Zeta-Probe GT membrane (BioRad, Bio-Rad Laboratories, Hercules, CA). Radioactive probes were prepared by random priming, using either full-length HBV DNA or 18S cDNA template and ^{32}P labelled αdCTP (Amersham). After hybridization the membrane was washed and exposed to X-Omat film (Kodak, Rochester, NY) at -80^oC.

2.3.2. Relative quantification by Real Time PCR

For each sample, 2μg RNA were reversed transcribed to cDNA in 20μl reactions using the RT^2 First Strand kit (Qiagen). The relative levels of pgRNA and total HBV RNA transcript concentrations were analysed by real time PCR with a TaqMan platform (Life technologies) in a Light-Cycler (Roche Diagnostic, West Sussex, UK). Each PCR reaction was performed in a 75μl reaction containing cDNA synthesized from 500ng of total RNA, 0.5μmol/L forward and reverse primers, 0.2μmol/L 3' FL-labelled probe, and 0.4μmol/L LC-labelled probe. The forward and reverse primers for the detection of pgRNA were 5′-GCCTTAGAGTCTCCTGAGCA-3′ and 5′–GAGGGAGTTCTTCTTCTAGG-3′, respectively, and the hybridization probes were 5’-AGTGTGGATTCGCACTCCTCCAGC-

FL-3' and LCRed640-5′ATAGACCACCAAATGCCCCTATCTTATCAAC-3′(Belloni et al., 2012). For the total HBV RNA expression the forward and reverse primers used were 5’-CTCGTGGTGGACTTCTCTC-3’ and 5’-CAGCAGGATGAAGAGGAA-3’, and the probes were 5’-LCRed640-TGTCCTGGTTATCGCTGGATGTGTCT-PH-3’ and 5’-CACTCACCAACCTCCTGTCCTCCAA-FL-3’. The h-GAPDH housekeeping gene Light Cycler Set (Roche DNA control kit, Roche Diagnostics) was used to normalize RNA expression.

For the detection of SIN3A, SUV39 and DNA methyltransferase 1 (DNMT1), real time PCR arrays were performed with customized RT^2 custom arrays containing pre-dispensed primer assays on a StepOne Plus Real Time PCR system (AB Applied Biosystems) using the RT^2 SYBR Green/qPCR Master Mix (Qiagen) and including two housekeeping genes (GAPDH and β-Actin) and three internal controls. Each PCR reaction contained cDNA synthesized from 125ng of total RNA. The thermocycler parameters were 95°C for 10min, followed by 45 cycles of 95°C for 15s and 60°C for 1min. Relative changes in gene expression for both viral and cell RNA transcripts were calculated using the $\Delta\Delta C_t$ (threshold cycle) method. Threshold cycle numbers $(Ct)^2$ above 35 were considered below detection level.

2.4. Purification and analysis of HBV DNA from transfections

HBV DNA was purified from the nuclei of HepG2 cells 48h post-transfection; washed once with ice-cold phosphate-buffered saline and digested overnight at 50°C with a buffer containing 0.9% NaCl, 10mM Tris-HCl pH8, 10mM EDTA, 0.5% SDS and 0.2mg/ml Proteinase K. DNA samples were then purified by phenol chloroform extraction (1:1) and ethanol precipitation in the presence of 20µg Glycogen (Invitrogen). DNA was further treated with 100µg/ml plasmid safe DNase I (Epicentre, Madison, WI) for 30min at 37°C and the reaction was stopped by incubation at 70°C for 45min. Following phenol chloroform extraction and ethanol precipitation, the DNA was resuspended in distilled water and stored at -20°C until used for Southern blotting.

To purify cytoplasmic HBV DNA, HepG2 transfected cells were lysed in 50mM Tris-HCl pH8 and 1mM EDTA, 1% NP-40. Sample preparations were incubated on ice for 30min and then centrifuged for 3min at 14,000rpm. The resulting supernatants were next incubated at 50°C for 2h with 0.2mg/ml Proteinase K and 1% SDS and then processed as above.

For Southern blotting, 7µg of cytoplasmic or nucleic DNA extracts were separated on a 0.8% ethidium-blomide agarose gel and blotted onto a Zeta-Probe GT membrane (BioRad, Bio-Rad Laboratories, CA). Radioactive probes were prepared by random priming using full-length HBV DNA template and ^{32}P labelled αdCTP (Amersham). After hybridization the membrane was washed and exposed to X-Omat film (Kodak) at -80°C.

2.5. Quantification of HBV cccDNA by Real Time PCR

Intracellular DNA was extracted from cell pellets by overnight digestion at 50°C with a Proteinase K buffer containing 0.9% NaCl, 10mM Tris-HCl pH8, 10mM EDTA, 0.5% SDS and 0.2mg/ml Proteinase K and were then treated with 100µg/ml plasmid safe DNase I (Epicentre, Madison, WI). DNA samples were then purified by phenol chloroform extractions followed by ethanol precipitation in the presence of 20µg of glycogen (Invitrogen). Real-time PCR was performed using 25µl containing 200ng DNA, 2xTaqMan reagent (Life technologies), 0.5µmol/L forward and reverse primers, and 0.2µmol/L FAM-labelled probe. Forward and reverse primers were 5'-CTCCCCGTCTGTGCCTTCT-3'

(HBVcccF nt1548-1563) and 5'-GCCCCAAAGCCACCCAAG-3' (HBVcccR nt1900-1883), respectively, and the hybridization probe was FAM-5'-CGTCGCATGGARACCACCGTGAACGCC-3' (HBV probe nt1602-1628). To normalize gene expression, β-globin amplification was performed by 5'-GGCAACCCTAAGGTGAAGGC-3' (Glob-F), 5'-GGTGAGCCAGGCCATCACTA-3' (Glob-R), and the hybridization probe was YAK-5'-CATGGCAAGAAAGTGCTCGGTGCCT-3' (Glob probe) (MacKenzie et al., 2001). Amplification of both cccDNA and β-globin was performed in a Light-Cycler (Roche, Basel, Switzerland) as follows: 95^{o}C for 10min then 45 cycles of 95^{o}C for 10 seconds, 60^{o}C for 5 seconds, 63^{o}C for 10 seconds, and 72^{o}C for 20 seconds. DNA from non-transfected cells was included in each experiment as negative control.

2.6. HBsAg detection by ELISA

Culture supernatants were tested for HBsAg by using a Murex HBsAg ELISA kit (Abbott Laboratories, Chicago, IL). HBsAg levels were detected semi-quantitatively by measuring the optical density at 450nm according to the manufacturer's instructions.

2.7. Chromatin Immunoprecipitation Assay

Chromatin ImmunoPrecipitation assays (ChiP) were performed as previously described (Pollicino et al. 2006). Briefly, 48h after transfection with 500ng SapI digested HBV monomers, HepG2 cells were crossed linked in 1% formaldehyde. The crossed-linked reaction was quenched with 50mM glycine for 5min. Cells were washed with PBS and pellets were resuspended in a Yosh A buffer and protease inhibitor cocktail. Sample preparations were sheared by sonication at 80% power in a 1% SDS lysis buffer to generate cellular chromatin fragments of 300-400bp. The chromatin was then subjected to immunoprecipitation for 14-16 h at 4^{o}C using antibodies specific to ^{Ac}H4 (rabbit polyclonal IgG antibody, Upstate, Charlottesville, Va, USA), HDAC1 (Rabbit polyclonal IgG antibody, Upstate), and DNMT1 (Rabbit Polyclonal IgG, Santa Cruz). Immunoprecipitation with non-specific IgG immunoglobulins (Santa Cruz Biotech) was included in each experiment as negative control. After the reverse cross-linking step, chromatin immunoprecipitates were treated with plasmid-safe DNase for 45 min at 37^{o}C to degrade contaminating HBV OC species and then subjected to PCR (50^{o}C/40 cycles) using forward and reverse primers P23F (5'-CTGAATCCTGCGGACGACCC-3', nt1441-1460) and P24R (5'-CCCAAGGCACAGCTTGGAGG-3', nt1889-1869), respectively. These primers are specific for the precore-core promoter region and discriminate between OC DNA present in virions and nuclear cccDNA (Pollicino et al., 2006). The synthesized fragments were separated on a 2% agarose gel and visualized with ethidium bromide.

2.8. Signal Intensity assessment

Autoradiography and PCR images were digitalised and analysed with a GS-800 Calibrated Densitometer (Biorad). Quantitative analysis of the signal intensity was performed with Quantity One 1-D Analysis Software (BioRad).

2.9. Methylation analysis

The methylation status of the cccDNA was investigated by bisulfite sequencing analysis at 48h following transfection of HepG2 cells with 500ng SapI digested HBV DNA monomers. Primers for bisulfite sequencing were designed to target a portion of CpG island II that was specific for the cccDNA region. DNA samples were isolated from transfections by overnight digestion at 50^{o}C with proteinase K buffer (described above), by phenol chloroform extraction and ethanol precipitation in the presence of 20μg of glycogen (Invitrogen) and treatment with 100μg/ml plasmid safe DNase I (Epicentre). From each transfection 500ng DNA was subjected to bisulfite conversion (EpiTect Bisulfite Kit, Qiagen) according to the manufacturer's instructions. The methylated cccDNA region (30ng DNA) was amplified by PCR using forward and reverse primers, 5'-GTTTGTGTTTTTTTATTTGTCGGATC-3' (cccMethF nt1551-1576) and 5'-CAATCTTTAAAATATACCTCAAAATCGA-3' (cccMethR nt1717-1690), respectively, at 50^{o}C/40 cycles. The PCR products were gel extracted and cloned into pCRII TA-vector (Invitrogen). For each transfection ten to twelve clones were selected for sequencing.

2.10. Statistical Analysis

Two group comparisons of continuous variables were performed using the non-parametric Mann-Whitney test with two tailed values (GraphPrism 6). P values below 0.05 were considered statistically significant.

3. RESULTS

3.1. HBV RNA transcripts are lower in transfections with BCP than in WT or Precore constructs

HBV transcript accumulation was assessed by Northern blot analysis and Real Time PCR quantification and data was acquired from three independent transfection experiments with duplicates (Figure 1). PgRNA levels were lower in the transfections with the BCP double (A1762T/G1764A) and triple mutated variants (A1762T/G1764A/C1766T) compared to the WT but these differences were not significant (p=0.1 and p=0.07, respectively) (Figure 1A-B), while the G1896A and G1896A/G1899A variants resulted in similar pgRNA levels. The RNA expression of the preS1 and preS2/S HBsAgs in BCP transfections was slightly lower for the BCP and G1899A mutations (Figure 1A-B). We used as an internal control a linear mutated HBV monomer bearing an in-frame deletion in the preS1 region. Northern blot analysis showed that the transfection with the preS1 mutant did not produce preS1 mRNA confirming the validity of the assay (Figure 1C). Total HBV RNA expression in the transfections with the A1762T/G1764A mutant was significantly decreased in comparison with the WT (P=0.02)(Figure 1D). Both A1762T/G1764A and A1762T/G1764A/C1766T constructs yielded less total HBV RNA levels than the G1896A (P=0.02), G1899A (P=0.02) and G1896A/G1899A variants (P=0.02) (Figure 1D). PgRNA expression was significantly decreased in transfections with the A1762T/G1764A/C1766T construct (P=0.02) while in A1762T/G1764A construct transfections this difference was not statistically significant (P=0.07). G1896A, G1899A and G1896A/G1899A variants gave similar pgRNA and total HBV RNA levels to the WT (Figure 1D). In combination, BCP mutations produced consistently lower levels of most viral transcripts while the precore mutations produced similar or slightly higher transcript levels.

3.2. Replication capacity of BCP constructs is defective in both cytoplasmic and nuclear compartments

Southern blot analysis of HBV DNA extracted from cytoplasmic and nuclear compartments was used to assess DNA replicative intermediate accumulation in transfected cells (Figure 2). The results were acquired from three independent transfection experiments and were run in duplicates. There were no significant statistical differences in HBV replication capacity between the WT and the mutated variants. However, we observed that in both cytoplasmic core particles and nuclear extracts, the BCP mutations caused decreased accumulation of

HBV OC, DS, SS DNA and cccDNA levels (P=0.07) (Figure 2A and B). The precore transfections produced similar levels of all replicative intermediates.

3.3. Detection of cccDNA by Real Time PCR

Measurement of cccDNA levels showed that these were lower in BCP construct transfected cultures than in WT or precore variant constructs (Figure 3A). These differences did not reach statistical significance. To determine whether this slight decrease was due to defective production of the HBeAg or HBx proteins, constructs expressing these proteins were co-transfected with BCP mutated ones. The presence of the HBe- and HBx- expressing plasmids did not change the cccDNA levels (Figure 3B). However, the addition of the epigenetic regulators TSA and curcumin induced a 2 to 3-fold increase in cccDNA accumulation in all transfections (cccDNA levels were increased from an average relative expression of 0.05 to 0.1 and 0.15, respectively) (Figure 3C-D). Results are shown from three independent transfection experiments with duplicates.

To determine the replication fitness of the mutants we calculated the ratios of pgRNA to cccDNA expression assessed by the RT PCR experiments. We found that the pgRNA/cccDNA ratio of the A1762T/G1764A and A1762T/G1764A/C1766T constructs was significantly lower than the WT (P=0.028) and the G1899A and G1896A/G1899A variants (P=0.028). However, these differences were not significant when we compared the ratios calculated by the optical densities data (Northern and Southern blots). This may be a limitation of the fogging of the films.

3.4. ELISA

HBsAg levels in culture media were assessed by ELISA, 48h post-transfection, from 22 independent experiments (Figure 4) relative to those of WT transfected cells for P value estimation**.** Transfections with A1762T/G1764A and A1762T/G1764A/C1766T mutants produced significantly lower HBsAg levels (P=0.006 and P=0.01, respectively). G1896A, G1899A and G1896A/G1899 variants led to the secretion of similar HBsAg levels to the WT.

3.5. Recruitment of H4 and HDAC1 onto cccDNA correlates with the presence of mutations

To investigate whether the defective replication efficiency of the BCP mutations correlated with the acetylation of cccDNA-histones we used the ChiP technique. The results were determined from three independent transfection experiments and were run in triplicate. All mutations decreased the acetylation of the H4 cccDNA-bound histone (Figure 5). The BCP mutations almost totally hampered H4 cccDNA-bound acetylation while they induced a 2-fold upregulation of HDAC1 recruitment onto cccDNA as compared to WT (Figure 5B). The precore mutants slightly increased HDAC1 expression while the WT virus blocked its expression. DNMT1 recruitment onto cccDNA was not affected by the presence of the mutations.

3.6. HBV cccDNA is a target for methylation

To determine if the differences in replication fitness and cccDNA production of the relevant mutations correlate with the methylation of cccDNA, we performed bisulfite sequencing analysis from three independent transfection experiments. The cccDNA region examined contained 6 CpG dinucleotides and 33 cytosines in the non-CpG context. We found that 2 out of 6 CpG dinucleotides and 1 out of 33 non-CpG cytosines were methylated (33.3% and

3% methylation, respectively) in all transfections independently of the construct used for transfection.

3.7. Expression of epigenetic regulators was not affected by the presence of mutations

We further assessed the RNA expression of three epigenetic regulators, SIN3A, SUV39 and DNMT1 from three independent transfection experiments in triplicate (Figure 6A-C). SIN3A is a transcriptional regulator that promotes histone deacetylation while SUV39 is a selective methyltransferase of H3 histone. DNMT1 maintains the methylation pattern and methylates the hemimethylated CpG islands. There were no significant differences observed in SUV39 levels between the cells transfected with WT, mutated constructs or non-transfected cells (Figure 6A-B). SIN3A expression was higher in transfections with the 1896 and 1899 precore variants but these differences did not reach statistical significance ($p=0.07$). DNMT1 expression was significantly higher in cells transfected with WT compared to non-transfected cells, while there was no significant changes between cells transfected with WT and mutated variants (Figure 6C).

4. DISCUSSION

In the course of CHB infection, HBeAg seroconversion is a turning point in the interplay between the virus and its host. The appearance of anti-HBe eventually reduces viral replication and is accompanied by the emergence of BCP and precore mutants that downregulate or abolish HBeAg and become the predominant variants (Okamoto et al., 1994; Buckwold et al., 1996;Moriyama et al., 1996). These variants may evade the immune response resulting in poor responses to IFN treatment, more severe liver disease and more rapid HCC progression overtime (Baptista et al., 1999; Kao et al., 2003; Munoz et al., 2011;Li et al., 2013). In this study, common BCP mutations resulted in relatively low viral transcription capacity, significantly lower pgRNA/cccDNA ratios and decreased virion release (HBsAg secretion) that concur with the expected lower HBeAg levels *in vivo*. The major precore mutations 1896, 1899 and 1896/99 did not affect viral transcription and replication capacity. Previous studies with these variants reached conflicting conclusions. A few groups showed that BCP mutants increased viral replication and were associated with an increase in pgRNA (Moriyama et al., 1996), with enhanced encapsidation (Buckwold et al., 1996), or with low HBx protein levels (Yu et al., 2012). Some studies reported no increase in viral replication (Scaglioni et al., 1997; Gunther et al., 1998; Chun et al., 2000; Yoo et al., 2003), whilst others demonstrated defective viral replication capacity (Jammeh et al., 2008; Fang et al., 2009; Li et al., 2013). The *in vivo* studies utilised HBeAg as a marker of viral replication. However, during HBeAg seroconversion, viral loads fluctuate significantly due to complex viral immune responses and therefore HBeAg levels cannot represent viral replication (Chu and Liaw, 2007; Liaw et al., 2010). Furthermore, cell based studies have employed plasmids containing either subgenomic mutated fragments isolated from different genetic backgrounds or infectious clones with the mutations generated by site-directed mutagenesis. HBV propagation with these constructs does not recapitulate the complete viral replication cycle in full as the cccDNA step is largely omitted being substituted by the plasmid (Pollicino et al., 2006). The strength of this study is that we used a well-characterized plasmid free HBV replication system that has not been applied before to study the epigenetic effect of HBeAg variants on viral replication. Previous studies using the same linear transfection system demonstrated that it allows the full recapitulation of the HBV replication cycle including the *de novo* formation of functional cccDNA (Gunther et al., 1995; Pollicino et al., 2006). Furthermore, the ability of linear HBV molecules to start a complete viral replication cycle was established at 48h post-transfection by the detection of the cytoplasmic accumulation of HBV transcripts, all HBV replicative intermediates, HBsAg release and cccDNA detection by real-time PCR (Gunther et al., 1995; Pollicino et al., 2006)

Our data suggests that the BCP mutated variants show decreased replication fitness demonstrated by a significant reduction of pgRNA, total HBV RNA and viral release as measured by HBsAg secretion and decreased ratios of pgRNA/cccDNA. The overlapping nature of the HBV open reading frames (ORFs) entails that a mutation can alter the expression of multiple proteins depending on its position. The double 1762/64 BCP mutation causes amino-acid substitutions at positions 130 and 131 of HBx (Lee et al., 2011). To examine whether the decreased replication fitness of the BCP variants was due to defective HBe or HBx protein production, we added HBe- or HBx- expressing plasmids to the BCP transfections. This addition did not affect cccDNA production (Figure 3B).

Acetylation and deacetylation of both histone and non-histone proteins and DNA methylation play a crucial role in regulating chromatin remodelling of cccDNA and viral gene transcription (Pollicino et al., 2006;Vivekanandan et al., 2008;Guo et al., 2009). Increased transcriptional activity of cccDNA and high viral replication correlates with the hyperacetylation of H3 and H4 histones in CHB patients and *in vitro* (Pollicino et al., 2006). Furthermore, administration of IFN-α inhibits HBV replication by an active epigenetic control of cccDNA and specifically by inducing the hypoacetylation of cccDNA-bound histones and the active recruitment of HDAC1 onto cccDNA (Belloni et al., 2012;Liu et al., 2013).

To study the epigenetic regulation of cccDNA in HBeAg variants, initially we used the deacetylase inhibitor TSA, a potent inhibitor of HDAC, and curcumin, a potent hypomethylating agent (Delcuve et al., 2012;Teiten et al., 2013). Both agents induced a 2 to 3- fold rapid increase in nuclear cccDNA accumulation. Interestingly, curcumin has been also shown to be a selective inhibitor of p300 histone acetylatransferase and therefore it could as well hamper cccDNA transcription (Marcu et al., 2006). Nevertheless, it can be postulated that a cellular function sensitive to epigenetic inhibitors is involved in cccDNA transcriptional control. To investigate this further, we examined the recruitment of acetylated H4 histone, deacetyltransferase HDAC1 and methyltransferase DNMT1 onto cccDNA molecules using the Chip assay. We found that the reduced capacity of BCP mutants to effect viral secretion and transcription correlated with the downregulation of H4 acetylation and increased HDAC1 recruitment onto cccDNA. The presence of the precore mutations resulted in higher H4 acetylation and decreased accumulation of HDAC1. The recruitment of cccDNA-bound DNMT1 was active in all transfections, indicating that cccDNA methylation did not associate with the differential viral transcription and release of the HBeAg variants, due to non-accessibility to regulatory transcription factors. The PCR primers that we have used to selectively detect cccDNA within the immunoprecipitated chromatin discriminate between the OC HBV DNA and cccDNA. The validity of our data is further confirmed by the absence of signal in the IgG negative control PCR amplifications.

Histone modifications work in conjunction with methylation of CpG islands to regulate gene expression (Gibney and Nolan, 2010). The methylation of episomal cccDNA in human tissue of CHB patients has been associated with impaired cccDNA mRNA synthesis capability and suppressed viral gene expression (Guo et al., 2009; Vivekanandan et al., 2010; Koumbi et al., 2015). It has been reported that DNMT1, DNMT2 and DNMT3 expression is upregulated in response to HBV, leading to viral methylation and decreased viral replication (Liu et al., 2009;Vivekanandan et al., 2010). We demonstrated that one third of the CpG islands within the cccDNA region were methylated independently of the presence of mutations. Our results are in agreement with previous studies showing that cccDNA is a target for methylation (Vivekanandan et al., 2010; Koumbi et al., 2015). CccDNA methylation was accompanied by increased levels of cellular DNMT1 expression and the recruitment of DNMT1 onto cccDNA. DNMT1 upregulation was not associated with viral production of the BCP and precore variants. In addition, we observed that the level of cccDNA methylation in the livers of CHB patients was not associated with serum viremia levels (data not shown). SIN3A is a transcriptional regulator that promotes histone deacetylation (Silverstein and Ekwall, 2005). The single precore mutations 1896 and 1899 induced a slight increase in SIN3 expression that could correlate with the decreased acetylation of cccDNA. SUV39 is a selective histone methyltransferase that has been associated with IFN-α inhibition of cccDNA transcription and its expression was not significantly altered by transfections with HBV genomes (Belloni et al., 2012). The latter finding is in agreement with the finding that DNMT1 recruitment onto cccDNA was similar between the HBV variants examined.

Altogether our data suggests that BCP mutations result in lower viral production, transcriptional capacity, and pgRNA/cccDNA ratios suggesting that the long-term damage to the liver may not be due to increased viral replication, but the result of accumulating mutations in the core region. In the absence of the tolerogenic effect of HBeAg in patients carrying the mutants studied here, the immune response is directed against the core protein forcing the virus to change. There ensues a constant battle between the immune system and the virus leading to cumulative liver damage. Our findings indicate that BCP may be capable of modulating the epigenetic control of cccDNA via the recruitment of HDAC1 onto cccDNA and the hypoacetylation of cccDNA-bound H4 histones (Table 2). Moreover, they appear to inhibit viral replication with similar epigenetic mechanisms to IFNα-induced cccDNA inhibition (Belloni et al., 2012). This picture in many ways reflects what is seen *in vivo*. Patients in the anti-HBe negative phase with disease reactivation and variants being the dominant virus, have in general lower levels of circulating HBV DNA than their HBeAg positive counterparts. This may indeed correlate with the immune status of the patient, something that cannot be recapitulated in the *in vitro* situation. Moreover, since HBV genotype C was used in the present study, it remains possible that that these findings may differ depending on genotype or subgenotype. In addition, the type of experiments described here have employed a single isolate of the virus with a stable sequence (excluding the intended mutations), which does not mimic the quasispecies nature of the virus with amino-acid substitutions predominantly affecting the nucleocapsid protein (Alexopoulou et al., 1997; Alexopoulou and Karayiannis, 2014). Such changes may represent immune escape variants or may have an effect on encapsidation and subsequent replication steps.

5. CONCLUSIONS

Hypoacetylation of cccDNA-bound histones and upregulation of cccDNA-bound HDAC1 associate with the reduced viral release of the BCP mutants and have been also directly correlated with low viral replication in CHB patients. HBV cccDNA is a target for methylation in HBV transfected cells independently of the presence of mutations and is accompanied by cellular DNMT1 upregulation. The identification of HDACs and histone acetyltransferases involved in the dynamic epigenetic processes of cccDNA control not only would advance our understanding of cccDNA transcription control, but should also provide potential therapeutic targets for selective inhibition of cccDNA transcription.

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Figure Captions

Figure 1. (A) Northern blot analysis of HBV transcripts. 10µg RNA were extracted from HepG2 cells after 48h of transfection with 500ng linear HBV DNA monomers carrying BCP or precore mutations as shown. (B) Densitometric quantification of pregenomic HBV mRNA (3.5kb), and preS1 (2.4kb) and preS2/S (2.1kb) mRNAs; data is presented as fold change to the WT and was collected from 3 independent transfection experiments and are expressed as mean ± SD. (C) Northern blotting of 10µg RNA extracted from HepG2 cells 48h post-transfection with 500ng linear HBV DNA preS1 mutant that does not express the preS1 transcript. (D) RT PCR was used for the detection of pgRNA and total HBV RNA. Data is presented from 3 independent experiments as relative ratios ± SD.

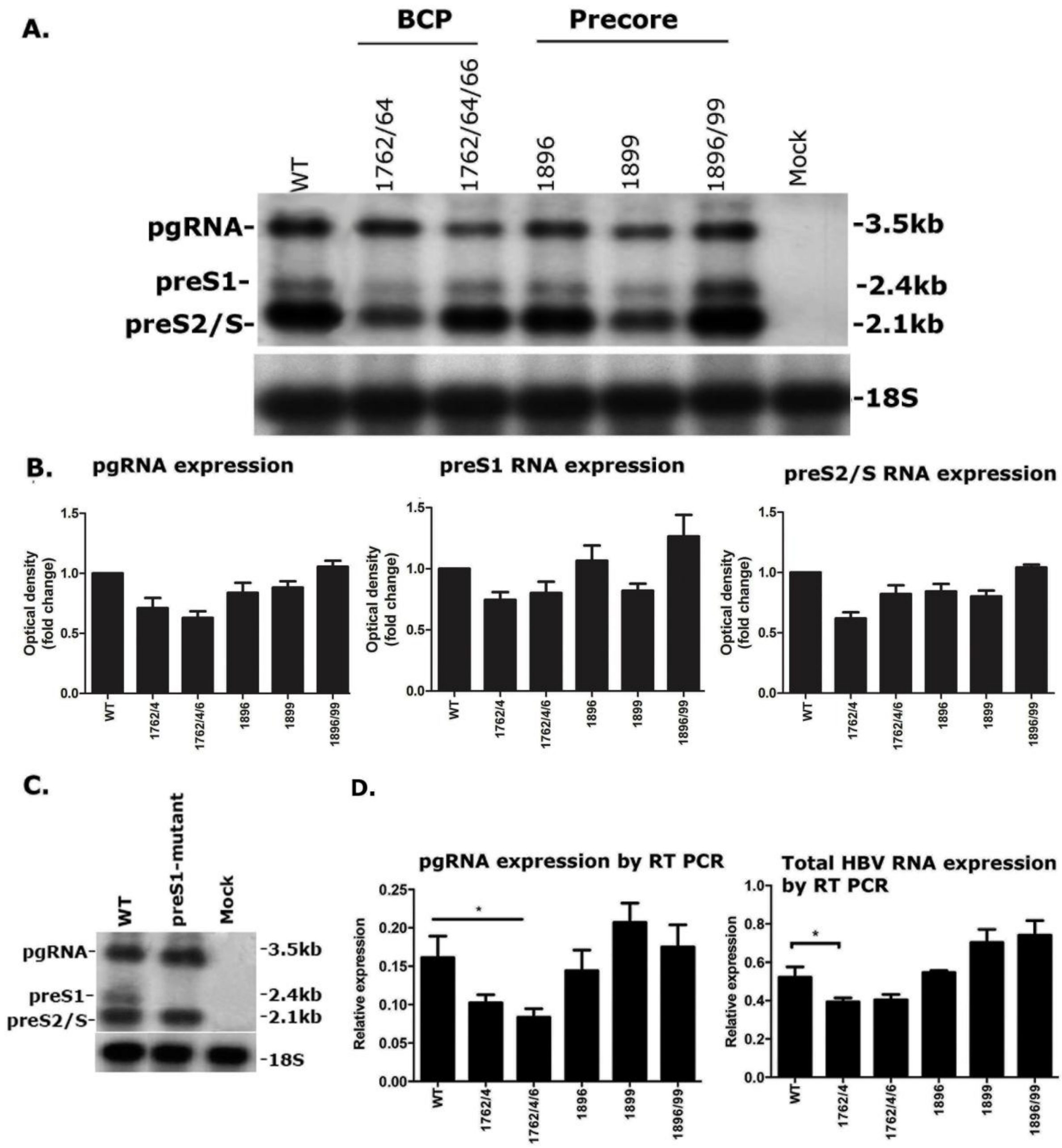


Figure 2. Replicative intermediates were detected by Southern blot hybridization. Lanes correspond to 7μg DNA extracted from (A) the cytoplasm and (B) the nucleus of HepG2 cells, which were transfected with 500ng SapI digested HBV monomers or 2μg of pUC19 as a mock control. Cells were harvested 48h post-transfection for Southern blotting and membranes were exposed to an X-ray film for autoradiography at -80 °C. (C) Densitometric quantification of HBV replicative intermediates, including open circular (OC), cccDNA, double stranded (DS) and single stranded (SS) DNA are shown. Data is presented as fold change compared to the WT and was collected from 3 independent experiments and optical densities are shown expressed as mean ± SD.

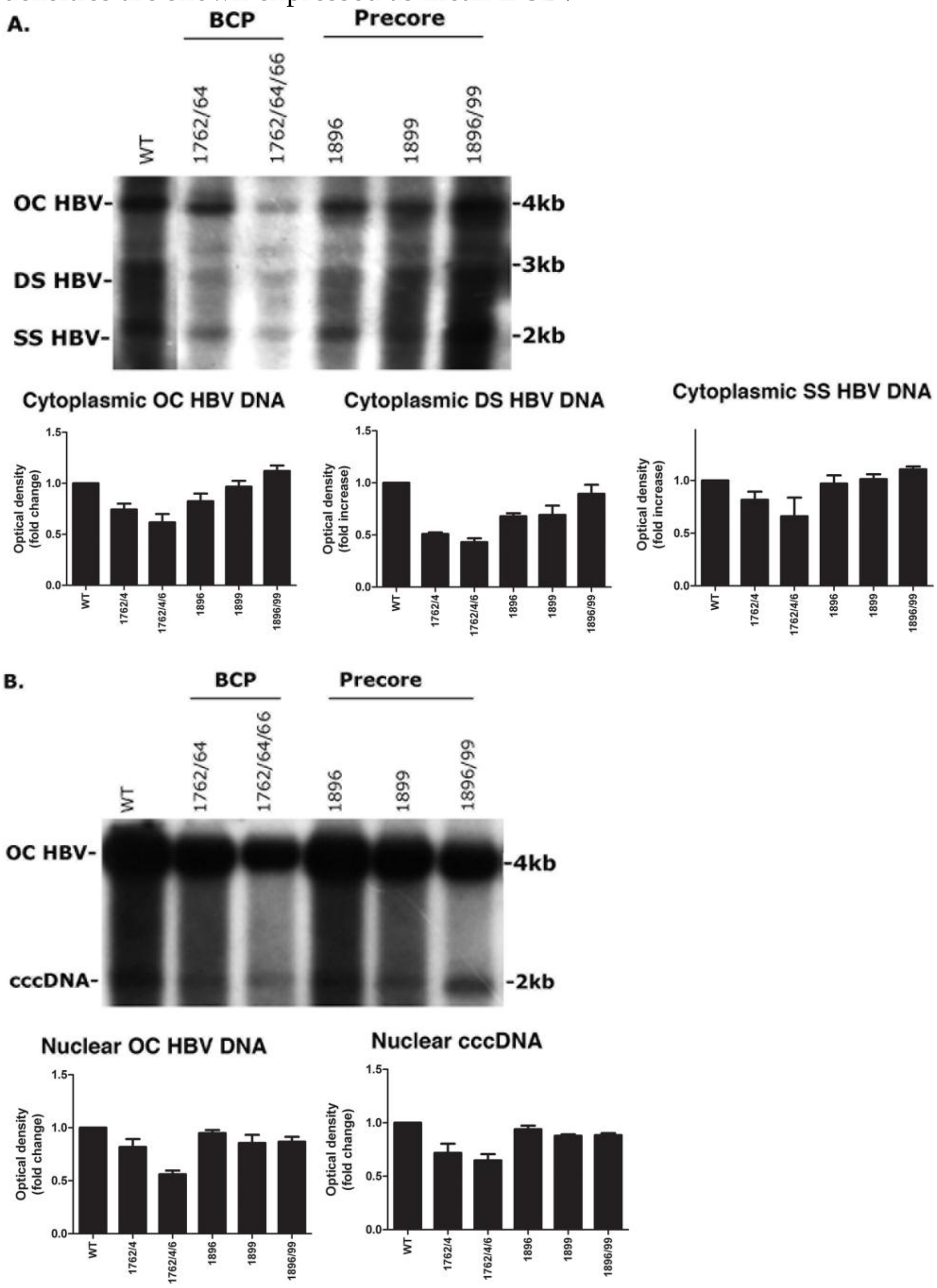


Figure 3. HBV cccDNA concentrations from nuclear extracts 48h after transfection with 500ng SapI digested HBV monomers were assessed by RT PCR. HBV cccDNA nuclear accumulation is presented from HepG2 transfected cells with 500ng SapI digested HBV DNA monomers: (A) WT and mutated constructs; (B) in the presence of HBe- or HBx-expressing plasmids and pDNA3.1 backbone as a mock control; and (C-D) in the presence of trichostatin (TSA) or curcumin, respectively. Data is presented from 3 transfection experiments; the RNA levels are relative to those of β-globin and presented as mean ± SD.

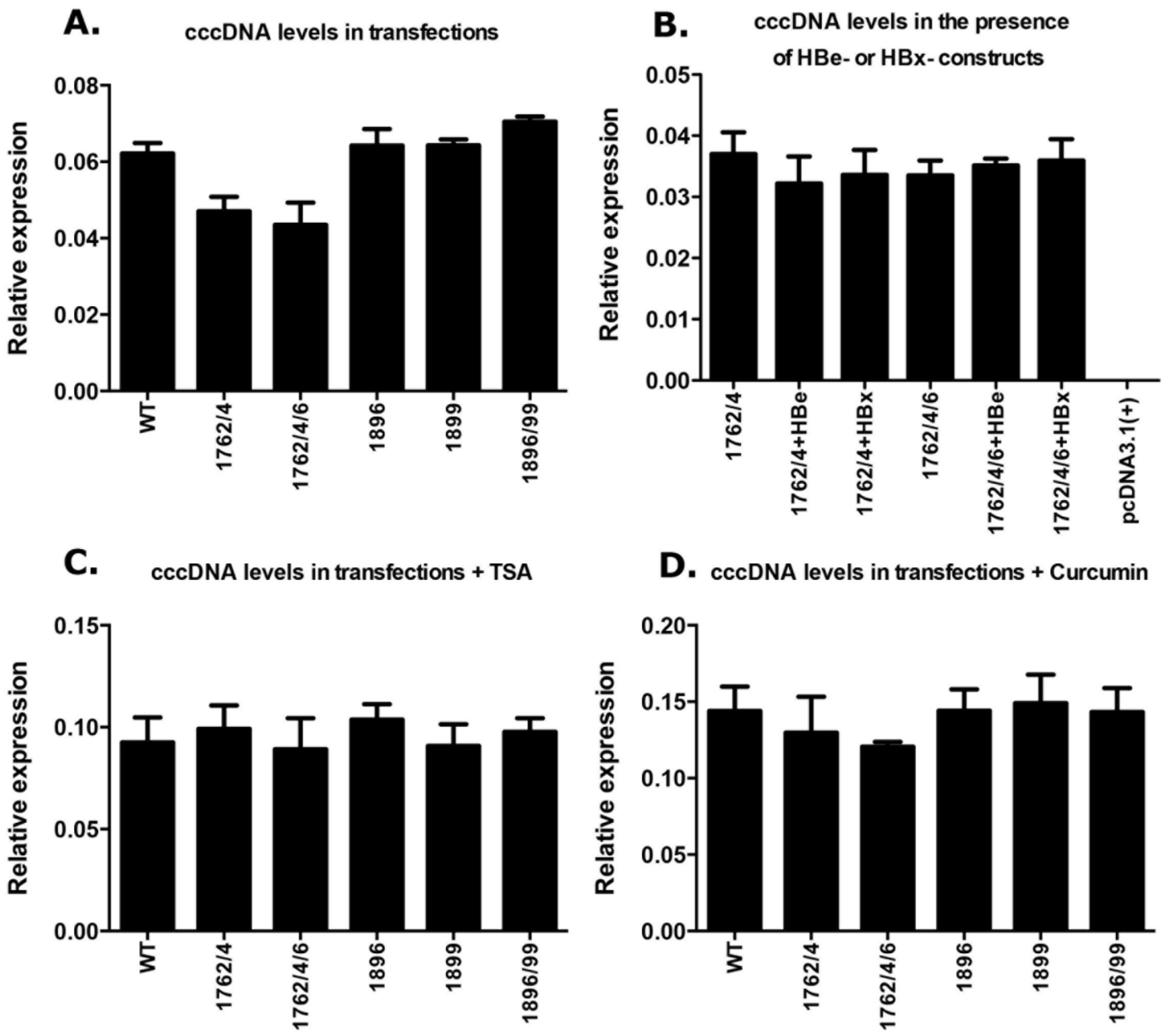


Figure 4. HBsAg release was detected 48h post-transfection in the supernatants of HepG2 cells transfected with 500ng HBV DNA monomers and was assessed semi-quantitavely by ELISA. Data is presented from 22 independent transfection experiments and shown as mean ± SD.

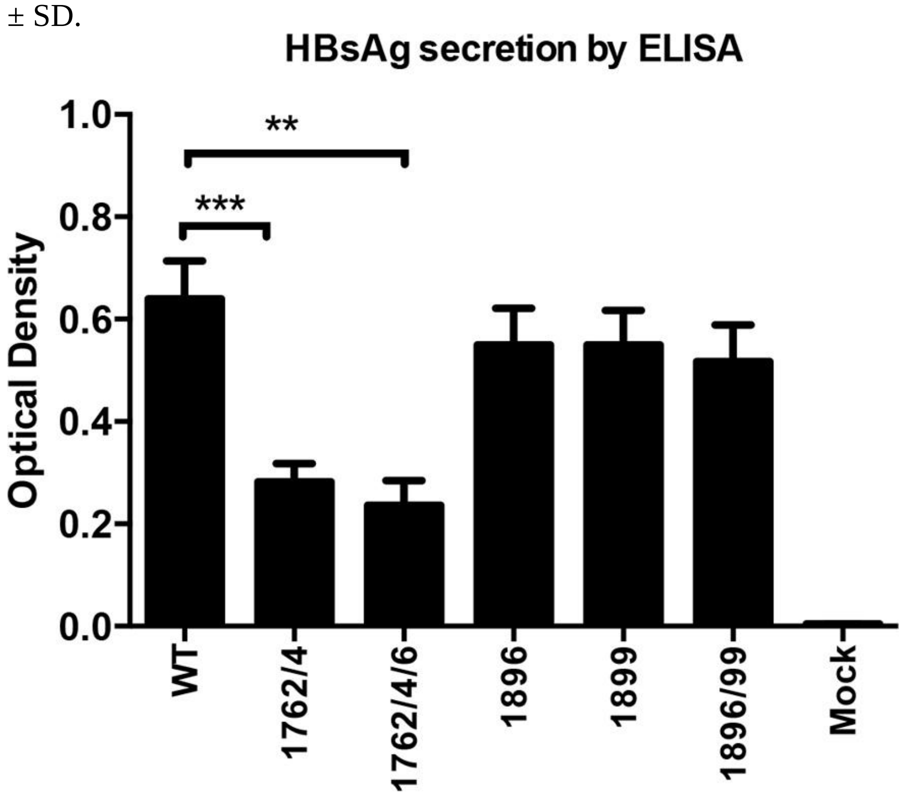


Figure 5. The recruitment of H4 histones, HDAC1 and DNMT1 onto cccDNA was assessed by ChiP assay. (A) ChiPs were performed 48h after HepG2 cells were transfected with 500ng SapI digested HBV monomers by using antibodies specific for ^{Ac}H4, HDAC1, DNMT1 or control IgG, followed by amplification (40 cycles) with PCR primers specific for cccDNA. Input refers to the PCR performed with the cccDNA-specific primers on the starting chromatin material from each experiment. (B) Densitometric quantification of signals acquired by PCR from 3 independent experiments and is expressed as mean ± SD. Data is presented as fold change to the WT.

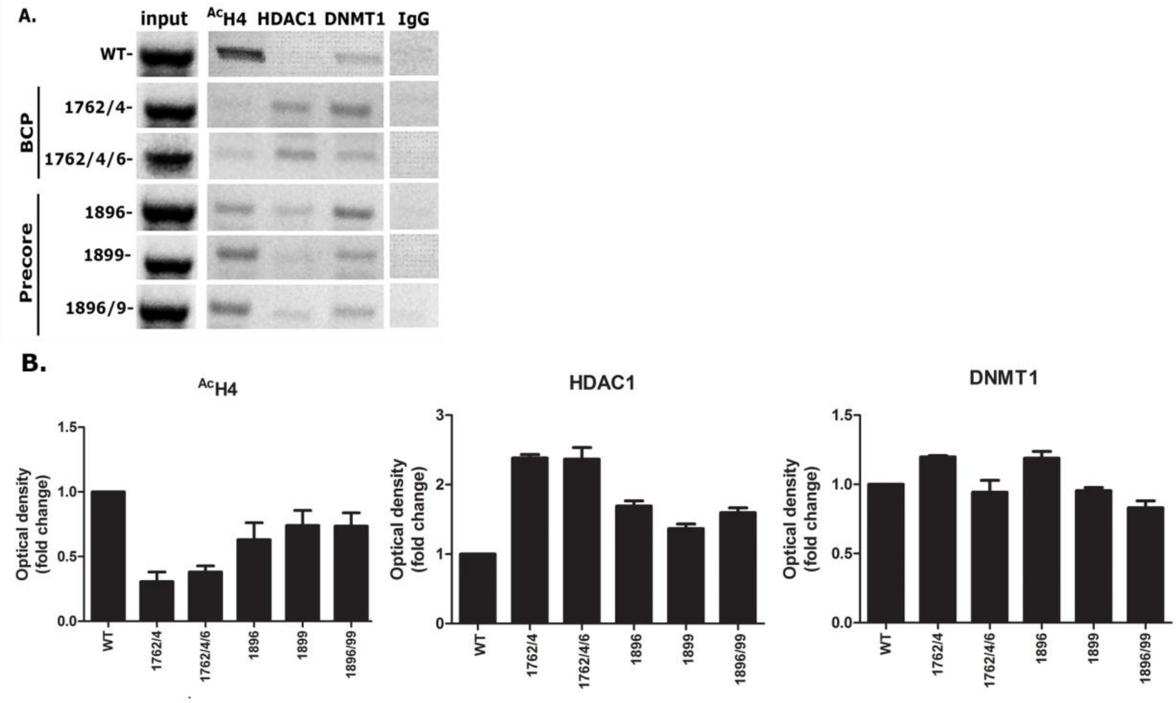
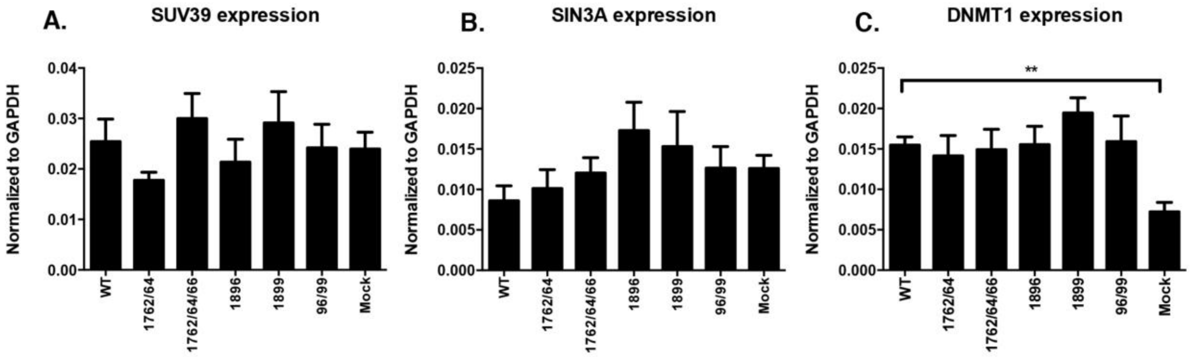


Figure 6. (A) RNA was extracted 48h after HepG2 transfection with 500ng SapI digested HBV DNA monomers and the levels of SUV39, (B) SIN3A, and (C) DNMT1 were assessed by RT PCR. RNA expression was normalized to h-GAPDH. Data is presented from 3 independent transfection experiments and values are expressed as relative ratios ± SD.



TABLES

Primers used for the generation of constructs used in transfections	
A1762T/G1764A -S	5'-GAGGAGATTAGGTTAA**T**G**A**TCTTTGTACTAGG
A1762T/G1764A-AS	5'-CCTAGTACAAAGA**T**C**A**TTAACCTAATCTCCTC
A1762T/G1764A/C1766T-S	5'GAGGAGATTAGGTTAA**T**G**A**T**T**TTTGTACTGGAG
A1762T/G1764A/C1766T-AS	5'-CTCCAGTACAAA**A**A**T**C**A**TTAACCTAATCTCCTC
G1896A-S	5'-GGGTGGCTTT**A**GGGCATGGACATTGAC
G1896A-AS	5'-GTCAATGTCCATGCCC**T**AAAGCCACCC
G1899A-S	5'-GGGTGGCTTTAGG**A**CATGGACATTGAC
G1899A-AS	5'-GTCAATGTCCATG**T**CCCAAAGCCACCC
G1896A/ G1899A-S	5'- GGGTGGCTTT**A**GG**A**CATGGACATTGAC
G1896A/ G1899A-AS	5'-GTCAATGTCCATG**T**CC**T**AAAGCCACCC
P1-S	5'-ccggaaagcttgagctcttcTTTTTCACCTCTGCCTAATCA
P2-AS	5'-CACCGGAAAGCTTGAGCTCTTCTTTTTcacctctgcctaatca
HBx-S	5'-TAAGCAgaattcATGGCTGCTAGGCTGT
HBx-AS	5'- TAAGCAtctagaATTAGGCAGAGGTGAAAAAGT
HBe-S	5'- TAAGCAgaattcATGCAACTTTTTCACCTCTG-3'
HBe-AS	5'- TAAGCAtctagaATTCCCAAAGCCACCCAA-3'

Table 1: Primers used full-length HBV genome generation by splice extension mutagenesis, insertion of *SapI* sites (lower case) and HBeAg-pcDNA3.1 and HBx-pcDNA3.1 plasmid construction. Mutated nucleotides are shown in bold.

		Effect of mutations compared to the WT HBV				
		BCP mutations		Precore mutations		
Experimental Methods	**Expression levels**	***1762/64***	***1762/64/66***	***1896***	***1899***	***1896/99***
Northern Blotting	pgRNA preS1 preS2/S	Low Low Low	Low Low Similar	Similar Similar Similar	Similar Low Low	High High High
Real Time PCR	Total HBV RNA pgRNA	Low Similar	Similar Low	Similar	High	High
Southern Blotting	Intracellular and Cytoplasmic HBV replicative intermediates	Similar	Similar	Similar	Similar	Similar
Real Time PCR	cccDNA	Similar	Similar	Similar	Similar	Similar
ELISA	HBsAg	Low	Low	Similar	Similar	Similar
ChiP	cccDNA bound -^{Ac}H4 histone -HDAC1 -DNMT1	 Low High Similar	 Low High Similar	 Similar Similar Similar	 Similar Similar Similar	 Similar Similar Similar
Methylation	cccDNA	Similar	Similar	Similar	Similar	Similar
RNA expression by RT PCR	SUV39 SIN3A DNMT1	Similar Similar Similar	Similar Similar Similar	Similar Similar Similar	Similar Similar Similar	Similar Similar Similar

Table 2: Summary of the main findings of the present study. **Abbreviations:** BCP, Basal Core Promoter; pgRNA, pregenomic RNA; ^{Ac}H4, acetylated H4 histone; HDAC1, class 1 histone deacetylase; DNMT1, DNA methyltransferase.