Hepatitis B virus X protein modulates remodelling of minichromosomes related to hepatitis B virus replication in HepG2 cells

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Abstract. Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) is organised into minichromosomes by histone and non-histone proteins. Remodelling of minichromosomes is crucial for the regulation of HBV replication, which is dependent on the presence of the hepatitis B virus X protein (HBx). However, the mechanisms of HBx-dependent HBV replication remain obscure. The objective of this study was to investigate the mechanism of HBx-dependent HBV replication through the pathway of chromatin remodelling. The role of HBx was investigated by transfecting human HepG2 cells with the linear full-length HBV genome (wild-type) or HBx-deficient mutant HBV DNA (HBx mutant). Our results showed that although the formation of cccDNA was not affected by HBx, HBV replication, transcription and antigen secretion were all significantly reduced, resulting from the absence of HBx. The acetylation, mono-methylation and phosphorylation of cccDNA-bound histone H3 were associated with HBV replication. In addition, the levels of cccDNA-bound methylated, phosphorylated and acetylated histone H3 decreased sharply in HBx mutant HBV DNA. HBx modulated not only the status of acetylation but also the methylation and phosphorylation of histone H3 bound to the cccDNA during HBV replication in HepG2 cells. These findings suggest that HBx plays an important role in modulating the remodelling of minichromosomes related to HBV replication and it may regulate viral replication through the pathway of chromatin remodelling.

Introduction

Hepatitis B virus (HBV) infection is a major risk factor for the development of severe liver diseases including hepatocellular carcinoma (HCC) (1). Hepatitis B virus X protein (HBx) is a 17-kDa protein encoded by the 3.2-kb HBV genome (2). Previous studies have indicated that HBx is oncogenic and can regulate HBV replication and transcription (3). One study was performed using a plasmid carrying a greater-than-unit-length HBV genome (payw1.2) (4) and a HBx-deficient plasmid containing a stop codon at amino acid position 7 (payw1.2.7) (5) transfected into HepG2 cells, respectively. Results have shown that the absence of HBx can induce a 65% reduction in HBV replication (6), and trans-complementation of HBx for the HBx-deficient plasmid can restore replication to wild-type levels (6-8). Another advanced study performed with the hydrodynamic injection of an HBx-deficient plasmid into mice showed the same results *in vivo* (6,9,10). These results demonstrated that HBx has an important role in modulating HBV replication.

However, to date, the mechanisms of HBx-dependent HBV replication are not very clear. HBV covalently closed circular DNA (cccDNA), the main replicative intermediate of HBV, is the template for transcription of all viral RNAs including pregenomic RNA (pgRNA) (11). Nuclear cccDNA, predominately from relaxed circular DNA (rcDNA) (12), is organised by histone and non-histone proteins into a viral minichromosome chromatin-like structure (11,13,14). Changes in the nucleosome (the basic unit of chromatin) structure and DNA-histone contacts may result in the remodelling of minichromosomes (14). Chromatin remodelling is closely associated with histone modifications, especially with modifications of histone H3 and

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Abbreviations: cccDNA, covalently closed circular DNA; ChIP, chromatin immunoprecipitation; CREB, cAMP-response element protein; DNMTs, DNA methyltransferases; DL, double-stranded linear; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescence protein; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e-antigen; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; IP, immunoprecipitation; IgG, immunoglobulin G; MAPK, mitogen-activated protein kinase; OD, optical density; pgRNA, pregenomic RNA; PVDF, polyvinylidene fluoride; PBS, phosphate-buffered saline; rcDNA, relaxed circular DNA; RIPA, radioimmunoprecipitation assay; SS, single-strand; SD, standard deviation

H4. These histone modifications may influence the structure of nucleosomes directly, and provide DNA binding sites for other proteins (13,15). Previous studies have shown that HBx can regulate HBV replication and transcription. Multiple signal transduction pathways and proteins may be involved in HBx-dependent HBV replication and transcription (16,17). These signalling mediators may have as their terminal target chromatin remodelling (18). Thus, it is hypothesized that the mechanisms of HBx-dependent HBV replication may involve the chromatin remodelling pathway.

Our previous study demonstrated that acetylation, methylation and phosphorylation of cccDNA-bound histone H3 occurs in HepG2 cells that are replicating wild-type HBV genome and that these histone modifications are associated with HBV replication (19). Our present study was designed to investigate the mechanism of HBx-dependent HBV replication through the pathway of chromatin remodelling. We established two *in vitro* replication models by transfecting human hepatoma HepG2 cells with the linear full-length HBV genome (wild-type) or the HBx-deficient mutant HBV DNA (HBx mutant) and investigated the regulation of HBx on replication, transcription and antigen secretion, and in particular, on the methylation, phosphorylation and acetylation of histone H3 bound to the cccDNA in chromatin during HBV replication in HepG2 cells.

Materials and methods

Plasmid. The plasmid pUC-HBV1.0, which contains full-length wild-type HBV genome and *Hin*dIII/*Sap*I and *Sac*I/*Sap*I restriction sites, was constructed as previously described (19). The HBx mutant plasmid pUC-HBV1.0.X7 that contains a stop codon (CAA-UAA) at amino acid 7 of HBx was derived from plasmid pUC-HBV1.0 by site-directed mutagenesis (5,8). Briefly, mutagenic primers were designed using primer design software developed by Stratagene. The forward primer was 5'-CTAGGCTGTGCTGCTAACTGGATCCTGCG-3' (mutated nucleotides underlined) and the reverse primer was 5'-CGCA GGATCCAGTTAGCAGCACAGCCTAG-3'. Using plasmid pUC-HBV1.0 as a template, the mutant products were amplified by the high-fidelity enzyme Premix PrimeSTAR® HS (Takara) through polymerase chain reaction (PCR). The PCR products were then digested with *Dpn*I enzyme (Fermentas), transformed into competent DH-5α cells, which were plated on LB plates containing ampicillin (100 µg/ml). Four white colonies selected randomly were prepared, and the plasmid DNA was extracted and digested with *Hin*dIII and *Sac*I enzymes (Takara). The plasmid, whose digested products were determined to be correct, was then sequenced (Takara) to confirm the mutation. All plasmids were prepared and purified using the Endotoxin-Free Plasmid Maxi kit (Tiangen Biotech, Co., Ltd.).

Cell culture and DNA transfection. Human hepatoma HepG2 cells were cultured in 6-well plates (Gibco) with high glucose DMEM containing 10% fetal calf serum (Hyclone) under 5% CO_2 at 37˚C. The linear full-length HBV genome and the HBx mutant HBV DNA were released from plasmids pUC-HBV1.0 and pUC-HBV1.0.X7, respectively, by *Sap*I enzyme (MBI) digestion and gel-purified by a DNA gel extraction kit (Promega). HBV DNA was transiently transfected into HepG2 cells using PolyJet™ reagent (SignaGen Laboratories). Briefly,

HepG2 cells were seeded at a density of $1.0x10^6$ cells in 6-well plates. Twenty-four hours later, cells at 70-80% confluence were transfected with HBV DNA (1.5 µg) and PolyJet™ reagent (4 µl). HepG2 cells were transfected with the linear full-length HBV genome (wild-type) or the HBx-deficient mutant HBV DNA (HBx mutant). A green fluorescent protein (GFP) expression vector (0.5 µg) was included in each transfection to assess transfection efficiency. After transfection, the cell culture medium was changed daily. The mean transfection efficiency was approximately 40%. A negative control with no plasmid transfected into the HepG2 cells was set up in each independent experiment.

Immunoprecipitation (IP) and western blot analysis detection of HBx. Cells were harvested at 48 and 96 h post-transfection and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS). Following centrifugation to remove cellular debris, the supernatants were incubated with Protein G Plus-Agarose (Santa Cruz Biotechnology, Inc.) and rabbit anti-HBx polyclonal antibody (Abcam) for immunoprecipitation. The precipitated complexes were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% bovine serum albumin (Sigma) for 1 h, the membrane was incubated overnight at 4˚C with rabbit anti-HBx polyclonal antibody (1:1,000), followed by incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:2,000; Pierce). The bound antibodies were visualised using an ECL chemiluminescence system (6,20).

Analysis of secreted HBV antigens. Culture supernatants collected from transfected cells at different time points were clarified by centrifugation at 3,000 rpm for 15 min and stored at -20˚C until used. Hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) were detected by an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai, KeHua) according to the manufacturer's instructions. The absorbance of the contents in each well was determined at the wavelength of 450 nm. Positive and negative control sera were included in each assay. The results were expressed as mean optical density (OD) values [mean ± standard deviation (SD)].

Southern blot analyses. At 48 and 96 h post-transfection, capsid-associated HBV DNA was extracted as described previously (8). Transfected cells were washed with cold phosphate-buffered saline (PBS) and lysed in 1% NP lysis buffer [50 mM Tris (pH 7.4), 1 mM EDTA, 1% NP-40, and 100 mM NaCl]. After centrifugation for 1 min at 12,000 rpm at 4˚C, the supernatants were treated with 100 µg/ml DNase I (Promega) for 30 min at 37˚C and then 0.5 mg/ml proteinase K at 50˚C for 2 h. Viral DNA released from lysed cores were extracted with phenol/chloroform, precipitated with ethanol, and dissolved in Tris-EDTA. Nuclear HBV cccDNA was extracted as described (15,21). Simply, transfected cells were lysed in cell lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2% NP-40, and 150 mM NaCl]. After centrifugation for 10 min at 12,000 rpm at 4˚C, the precipitate was resuspended in nuclear lysis buffer (6% SDS, 100 mM NaOH) and incubated for 30 min at 37˚C. The lysates were then neutralised with potassium acetate

(pH 4.8) and centrifuged for 10 min at 12,000 rpm at 4˚C. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation. The replicative intermediates of capsid-associated HBV DNA and nuclear HBV cccDNA were detected by Southern blotting. Ten micrograms of capsid-associated HBV DNA and HBV cccDNA was separated on a 1% agarose gel, transferred onto a positively charged nylon membrane, and hybridised with a ^{32}P-labelled full-length HBV DNA probe (Takara). The membrane was then washed and exposed to film at -80˚C (22).

Quantitative analysis of capsid-associated HBV DNA. At 24, 48, 72 and 96 h after transfection, capsid-associated HBV DNA in HepG2 cells was extracted and quantitated by real-time PCR using TaqMan probes. The primers were 5'-AGAAACAACA CATAGCGCCTCAT-3' (forward) and 5'-TGCCCCATGCTG TAGATCTTG-3' (reverse). The TaqMan probe was 5'-FAM-TGTGGGTCACCATATTCTTGGG-TAMER-3' (6). The cycling parameters, performed with an Applied Biosystems 7300 sequence detection system, were as followed: 95˚C for 30 sec, then 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. The plasmid pUC-HBV1.0 was diluted over a range of 10^9-10^3 copies and used as a standard. The results were expressed as the number of DNA copies/cell (mean ± SD).

Quantitative analysis of HBV cccDNA. HBV cccDNA was quantified by real-time PCR as previously described (15). HBV cccDNA extracted from transfected HepG2 cells was treated with Plasmid-Safe DNase (Epicentre) at 37˚C for 1 h to remove the open circular duplex HBV DNA and single-strand HBV DNA. The primers were 5'-CTCCCCGTCTGTGCCTTCT-3' (forward) and 5'-GCCCCAAAGCCACCCAAG-3' (reverse). The probes were 5'-GTTCACGGTGGTCTCCATGCAA CGT-FAM-3' and 5'-ROX-AGGTGAAGCGAAGTGCACAC GGACC-PO4-3' (15,19). Real-time PCR experiments were performed with a LightCycler (Roche) as followed: pre-denaturation for 10 min at 95˚C, then 45 cycles of 10 sec at 95˚C, 5 sec at 58˚C, 10 sec at 63˚C, and 20 sec at 72˚C. Standard curves were prepared as described in the quantitative analysis of capsid-associated HBV DNA by real-time PCR. The results were expressed as the number of DNA copies/cell (mean ± SD).

Quantitative analysis of HBV pregenomic RNA (pgRNA). For pgRNA analysis, total cellular RNA was extracted with TRIzol reagent (Invitrogen) from transfected HepG2 cells at different time points post-transfection. RNA concentration and purity were determined by ultraviolet spectrometry. The RNA samples were treated with RNase-Free DNase (Promega) at 37˚C for 30 min, and reverse transcribed into cDNA using PrimeScript® RT reagent kit (Takara). Each cDNA was quantified by real-time PCR using SYBR® Premix Ex Taq™ II kit (Takara). The specific primers for pgRNA were 5'-GCCTTAGAGTCTCCTGAGCA-3' (forward) and 5'-GAGGGAGTTCTTCTTCTAGG-3' (reverse) (21), and for GAPDH were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse). Amplification of GAPDH cDNA was used to normalise the RNA samples.

cccDNA chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed with an EZ-Magna ChIP assay

kit (no. 17-408; Millipore) according to the manufacturer's specifications. Briefly, at 24, 48, 72 and 96 h post-transfection, HepG2 cells were cross-linked by incubation with 1% formaldehyde for 10 min at room temperature, which was terminated with 10X glycine by incubation at room temperature for 5 min. The collected cells were washed twice with cold PBS and lysed with cell lysis buffer by incubation on ice for 15 min. The cell pellet was then resuspended in nuclear lysis buffer and sonicated to generate 300-400 bp DNA fragments. After centrifugation, 50 µl of each of the supernatants ($1x10^6$ cell equivalents) was diluted 1:10 with ChIP dilution buffer and a 1% volume of the mixture was taken as input. The chromatin was then subjected to immunoprecipitation for 14-16 h at 4˚C with anti-H3 (no. 06-755), anti-acetylated histone H3 at lysines 9 and 14 (no. 06-599), anti-monomethylated histone H3 at lysine 4 (no. 07-436), anti-phosphorylated histone H3 at serine 10 (no. 04-817; all were from Millipore) antibodies, and 20 µl of fully suspended protein A magnetic beads. Immunoprecipitation with the relevant nonspecific immunoglobulin G (IgG) was included in each experiment as a negative control. After reversal of the cross-linking, DNA from the antibody-bound and input fractions was isolated and treated with plasmid-safe DNase at 37˚C for 1 h. Purified ChIP cccDNA and input DNA were then analysed by PCR and real-time PCR using cccDNA-selective primers and probes (15). HBV cccDNA-selective primers were HBV P23 (5'-CTGAATCCCGCGGACGACCC-3') (1443-1462), and P24 (5'-ACCCAAGGCACAGCTTGGAGG-3') (1891-1871), which were specific to the HBV precore-core promoter region to distinguish cccDNA from rcDNA (25,26). The PCR reaction was performed with the high-fidelity enzyme Premix PrimeSTAR® HS as follows: 35 cycles of 10 sec at 98˚C, 5 sec at 60˚C and 1 min at 72˚C. The PCR products were analysed by electrophoresis. ChIP cccDNA and input DNA were absolutely quantified by real-time PCR as described above. Results were expressed as the percentage of input DNA.

Statistical analysis. The data presented from at least 3 separate experiments were expressed as the means ± SD. Statistical comparisons of the continuous variables between the 2 groups were performed using the nonparametric Wilcoxon rank-sum test (SPSS 19.0 software). P-values of <0.05 were assigned to indicate statistically significant results.

Results

HBx is required for enhancement of HBV replication in HepG2 cells. To detect the effect of HBx on HBV replication, equivalent number of HepG2 cells were transfected with the linear full-length HBV genome (wild-type) or the HBx mutant. Capsid-associated HBV DNA was extracted from HepG2 cells at 24, 48, 72 and 96 h post-transfection and quantified by real-time PCR. The levels of capsid-associated HBV DNA from the wild-type HBV-transfected cells were 39.1±2.9 copies/cell at 48 h which declined to 10.5±1.4 copies/cell at 96 h, while levels from the HBx mutant-transfected cells were 36.6±2.5 copies/cell at 48 h which decreased to 4.9±1.1 copies/cell at 96 h (Fig. 1A). Between 24 and 48 h, when HBV replication reached peak levels, the levels of capsid-associated DNA in the HBx mutant-transfected cells were slightly affected by the lack of
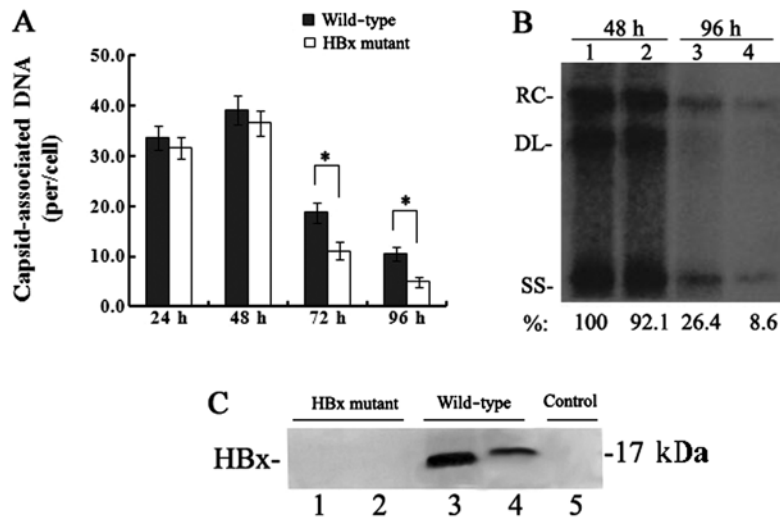
Figure 1. Effect of HBx on HBV replication in transfected HepG2 cells. (A) Quantification of capsid-associated HBV DNA by real-time PCR at 24, 48, 72 and 96 h post-transfection. Results are expressed as the number of capsid-associated DNA copies per cell (mean ± SD) from 3 independent experiments. Statistical significance is designated with asterisks above the brackets. (B) Southern blot analysis of capsid-associated viral DNA replicative intermediates at 48 and 96 h after transfection. Signal intensity of the single-stranded (SS) band underneath the double-stranded linear (DL) HBV DNA band was quantified with Quantity One Analysis software (Bio-Rad). The band corresponding to the DL HBV DNA was not included in the quantitative analysis, as this DNA might be partially derived from transfected input DNA. Lanes 1 and 3, the wild-type HBV genome; lanes 2 and 4, HBx mutant HBV DNA. The number at the bottom of each lane represents the relative levels of HBV DNA replicative intermediates, with those levels detected in the wild-type HBV-transfected cells at 48 h set to 100%, and levels measured in the HBx mutant-transfected cells are compared to those in the wild-type HBV-transfected cells from three independent experiments; RC, relaxed circular; DL, double-stranded linear; and SS, single-stranded forms. (C) Representative IP/western blotting. A negative control with no plasmid transfected into HepG2 cells (lane 1); HepG2 cells transfected with the linear HBx-deficient mutant HBV DNA (HBx mutant) and the full-length HBV genome (wild-type) were harvested at 48 and 96 h, and analysed by IP/western blotting for HBx protein. Lanes 1 and 3, 48 h; lanes 2 and 4, 96 h.

HBx (P>0.05); however, the levels of capsid-associated HBV DNA were significantly reduced at 72 and in particular at 96 h, which showed a 50-70% reduction (P<0.05) as compared to those levels in the wild-type HBV-transfected cells. The results of the Southern blotting by which replicative intermediates of capsid-associated DNA were detected with a ^{32}P-labelled full-length HBV DNA probe were consistent with those of the real-time PCR quantitation of capsid-associated DNA (Fig. 1B). A sensitive IP/western blot assay was used to detect the expression of HBx in transfected HepG2 cells. HBx was below the limit of detection by IP/western blot assay in the HBx mutant-transfected cells, but was detected at both 48 and 96 h in the wild-type HBV-transfected cells, with the expression level of HBx at 96 h much lower than that at 48 h (Fig. 1C). Together, these results demonstrate that HBx is required for the enhancement of HBV replication in HepG2 cells.

HBx affects the secretion of the HBV antigen. To investigate the effect of HBx on antigen secretion, the secretion of HBsAg and HBeAg from cell culture supernatants was tested by ELISA at 4 time-points. The results showed that the secretion of HBV antigens was detected in cells transfected with both wild-type and HBx mutant HBV DNA. At 24 h after transfection, there was no significant difference in the secretion of HBsAg and HBeAg between the 2 types of HBV DNA (P>0.05) (Fig. 2). With the extension of time after transfection, differences gradually appeared. In particular, at 96 h the secretion of HBsAg and HBeAg from HBx mutant-transfected cells was 55.7% (P<0.05) (Fig. 2A) and 55.2% (P<0.05) (Fig. 2B), respectively, of that from wild-type HBV-transfected cells. These results indicate that the lack of HBx reduces the secretion of viral antigens in HepG2 cells.

HBx does not affect HBV cccDNA formation, but upregulates the transcription of pgRNA. The HBV cccDNA extracted from HepG2 cells at 24, 48, 72 and 96 h post-transfection was purified after treatment with plasmid-safe DNase and quantitatively analysed by real-time PCR. The level of cccDNA in wild-type HBV-transfected cells was 38.4±3.1 copies/cell at 48 h which decreased to 6.9±1.5 copies/cell at 96 h, and the level in the HBx mutant-transfected cells was 36.5±2.7 copies/cell at 48 h which declined to 5.8±1.2 copies/cell at 96 h (Fig. 3A). When HBV replication reached a peak between 24 and 48 h or when levels of both nuclear cccDNA and HBV replication were reduced severely at 72 and 96 h, the mean copies of cccDNA per HepG2 cell were similar with the 2 types of HBV genome. The results of the real-time PCR quantitation of HBV cccDNA were confirmed by Southern blotting by which nuclear cccDNA was detected (Fig. 3B). Altogether, these results demonstrate that HBx is not required for HBV cccDNA formation.

Since HBx is not required for the formation of HBV cccDNA, the effect of HBx on a downstream step, pgRNA transcription, was investigated. At 4 time-points post-transfection, the extracted RNA was reverse transcribed and quantified by real-time PCR. These results showed that levels of pgRNA were slightly lower between 24 and 48 h after transfection in the HBx mutant DNA (P>0.05). However, differences gradually appeared after this time. In particular, at 96 h, the levels of pgRNA were reduced by 50-70% in the absence of HBx, compared to levels in the cells transfected with wild-type HBV DNA (P<0.05) (Fig. 3C). Taken together, these findings indicate that HBx upregulates pgRNA transcription without affecting the formation of HBV cccDNA.
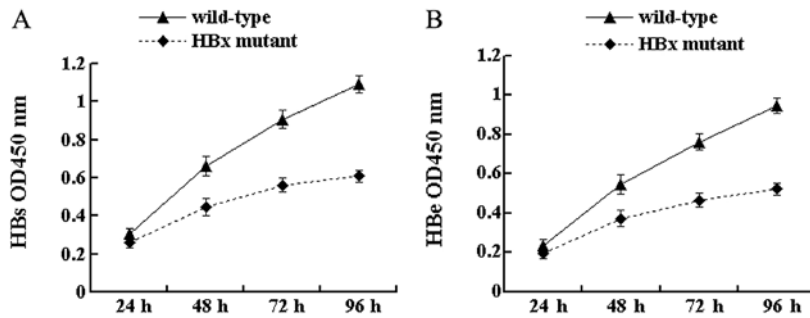
Figure 2. Effect of HBx on HBV antigen secretion in HepG2 cells transfected with the linear full-length HBV genome (wild-type) or the HBx-deficient mutant HBV DNA (HBx mutant). Culture supernatants were collected at 24, 48, 72 and 96 h after transfection and detected by ELISA. (A) HBsAg; (B) HBeAg. Results are expressed as the OD values (mean ± SD) measured at a wavelength of 450 nm from 3 independent experiments. SD, standard deviation.
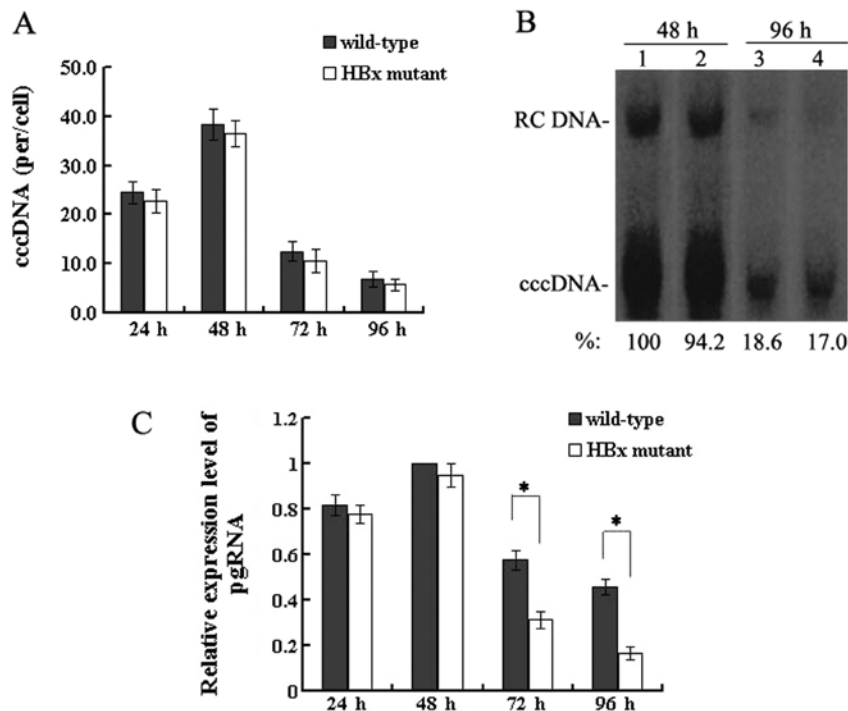

Figure 3. Effect of HBx on cccDNA formation and transcription of pgRNA in HepG2 cells transfected with the linear full-length HBV genome (wild-type) or the HBx-deficient mutant HBV DNA (HBx mutant). (A) Quantification of HBV cccDNA by real-time PCR at 4 time-points post-transfection. Results are expressed as the number of cccDNA copies/cell (mean ± SD) from 3 independent experiments (B) Southern blot analysis of nuclear cccDNA at 48 and 96 h after transfection. Signal intensity of the cccDNA band was quantified with Quantity One Analysis software. Lanes 1 and 3, the wild-type HBV genome; lanes 2 and 4, HBx mutant HBV DNA. The number at the bottom of each lane represents the relative levels of cccDNA, with those detected in the wild-type HBV-transfected cells at 48 h set to 100%, and levels measured in the HBx mutant HBV-transfected cells are compared to those in the wild-type HBV-transfected cells from 3 independent experiments. (C) Specific primers were used to quantitate relative HBV pregenomic RNA (pgRNA), and GAPDH amplification was used to normalise for each RNA sample. The level of pgRNA detected in the wild-type HBV-transfected cells at 48 h post-transfection was set to 1.0, and the level measured in HBx mutant HBV-transfected cells was compared to that in the wild-type HBV-transfected cells. Results are expressed as the mean ± SD from 3 independent experiments. Statistical significance is designated with asterisks above the bracket. pgRNA, pregenomic RNA.

HBx regulates remodelling of the minichromosome related to HBV replication. Since HBx has proven to be crucial for HBV replication and transcription, we sought to study whether HBx affects remodelling of the minichromosome related to HBV replication. Immunoprecipitated cccDNA from HepG2 cells at 24, 48, 72 and 96 h after transfection was amplified by PCR and quantified by real-time PCR. In cells transfected with the wild-type HBV genome (Fig. 4), the cccDNA-bound histone H3 was highly acetylated, hypermethylated and hyperphosphorylated simultaneously between 24 and 48 h post-transfection when the level of HBV replication reached a

peak. Levels of cccDNA-bound acetylated, mono-methylated and phosphorylated histone H3 peaked at 4.53±0.71, 3.31±0.62 and 3.62±0.59, in units of % input DNA, respectively. At 72 h, when the HBV replication declined, the levels of cccDNA-bound acetylated and phosphorylated histone H3 were sharply reduced compared to those at 48 h (all P<0.05). Both the acetylation and phosphorylation of cccDNA-bound H3 histone decreased by 72 h to 2.24±0.31 and 1.71±0.22 in units of % input DNA, respectively (all P<0.05). However, levels of cccDNA-bound methylated H3 did not change appreciably at 72 h, and the values in units of % input DNA were
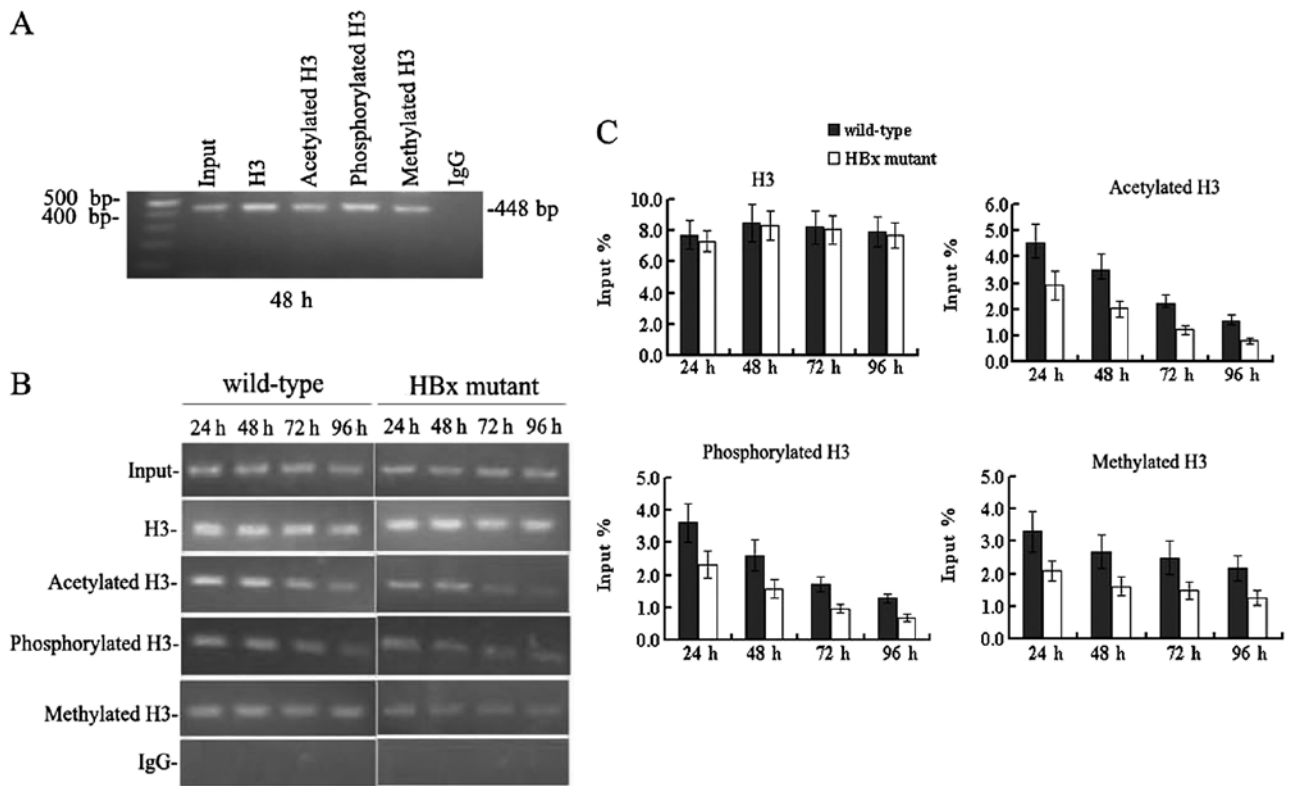
Figure 4. Regulation of HBx on the remodelling of the minichromosome related to HBV replication. (A) HepG2 cells were transfected with the linear full-length HBV genome (wild-type). Chromatin prepared at 48 h post-transfection was immunoprecipitated with the relevant control IgG or the specific anti-H3, anti-acetylated histone H3 at lysines 9 and 14, anti-monomethylated histone H3 at lysine 4 and anti-phosphorylated histone H3 at serine 10 antibodies, and analysed by semi-quantitative PCR. (B and C) HepG2 cells were transfected with the linear full-length HBV genome (wild-type) or the HBx-deficient mutant HBV DNA (HBx mutant). Chromatin prepared at 24, 48, 72 and 96 h post-transfection was immunoprecipitated with the relevant control IgG or specific anti-H3, anti-acetylated histone H3 at lysines 9 and 14, anti-monomethylated histone H3 at lysine 4 and anti-phosphorylated histone H3 at serine 10 antibodies, and analysed by (B) semi-quantitative PCR and (C) real-time quantitative PCR with HBV cccDNA-selective primers. The results are expressed as percentages of input chromatin (mean ± SD) from 3 independent experiments.

2.69 ± 0.52 and 2.49 ± 0.51 at 48 and 72 h, respectively (P>0.05) (Fig. 4B and C). These results indicate that the acetylation and phosphorylation of cccDNA-bound histones H3 are dynamic, but the methylation of cccDNA-bound histone H3 is relatively stable at lysine 4. Acetylation, methylation and phosphorylation of cccDNA-bound histone H3 paralleled HBV replication in HepG2 cells.

Next we investigated the epigenetic changes in cells transfected with the HBx mutant HBV genome. cccDNA-bound histone H3 was not only rapidly hypo-acetylated but also hypo-phosphorylated and hypomethylated (Fig. 4B and C). At 48 h post-transfection, cccDNA-bound acetylated, phosphorylated and methylated histone H3 were reduced by 40-50% in the absence of HBx compared to those levels in cells transfected with the wild-type HBV DNA (all P<0.05). The acetylation, methylation, and phosphorylation of cccDNA-bound H3 were 2.01 ± 0.32, 1.61 ± 0.28 and 1.58 ± 0.29 in units of % input DNA, respectively. At 96 h, levels of the above-mentioned modifications of histone H3 were very low. These results were in agreement with the reduction observed in HBV replication (Fig. 1A and B), pgRNA transcription (Fig. 3C) and antigen secretion (Fig. 2). Together, these findings demonstrate that HBx can affect the methylation, phosphorylation and acetylation of cccDNA-bound histone H3 during HBV replication in HepG2 cells. In other words, HBx can regulate the remodelling

of the minichromosomes related to HBV replication in HepG2 cells. Considering that epigenetic modifications of cccDNA-bound H3 histone parallel HBV replication, it is now possible to state that HBx may regulate viral replication through the pathway of chromatin remodelling.

Discussion

In our study, the HBx-deficient plasmid pUC-HBV1.0.X7 was successfully constructed by site-directed mutagenesis, and two *in vitro* replication models by transfecting HepG2 cells with the linear full-length HBV genome (wild-type) or the HBx-deficient mutant HBV DNA (HBx mutant) were established successfully. We found that although the formation of HBV cccDNA was not affected by HBx, there was a dramatic reduction in HBV replication, pgRNA transcription and antigen secretion in the absence of HBx compared to levels in cells transfected with the wild-type HBV genome. In addition, the levels of cccDNA-bound methylated, phosphorylated and acetylated histone H3 decreased sharply in HBx mutant HBV DNA. These results suggest that HBx is required for the enhancement of HBV replication and transcription. HBx modulates not only the status of acetylation but also the methylation and phosphorylation of cccDNA-bound histone H3 related to HBV replication in HepG2 cells.

Although it is not completely clear why HBV cccDNA formation is not affected by HBx, our study has demonstrated that HBx plays an importance role in regulating methylation and phosphorylation in addition to acetylation of cccDNA-bound histone H3 during HBV replication and elucidated the mechanism of HBx-dependent HBV replication through the pathway of chromatin remodelling. It has been shown that HBx does not directly bind to DNA sequences but is recruited onto the chromatin through its ability to interact with various cellular partners and proteins (23). The recruitment of HBx onto the cccDNA parallels the dynamic changes of cccDNA-bound acetylated H3 (21). HBx favours the aceylation of histone H3 bound to cccDNA and modulates HBV replication and transcription (15,24). However, our present study has shown that the methylation, phosphorylation and acetylation of cccDNA-bound histone H3 paralleled HBV replication in HepG2 cells. The cccDNA-bound histone H3 was highly acetylated, hypermethylated and hyperphosphorylated when the level of HBV replication reached a peak; while the levels of cccDNA-bound acetylated and phosphorylated histone H3 were also reduced when the HBV replication declined, although the levels of cccDNA-bound methylated H3 did not change appreciably. But why acetylation and phosphorylation of cccDNA-bound histone H3 decreased over time apart from methylation remains obscure. The difference in HBV replication between the groups of wild-type and HBx-mutant appeared only at 72 h post-transfection whereas the difference in epigenetic modifications was already detected 24 h post-transfection. The possible interpretations are that the absence of HBx may change chromatin structure and DNA-histone contacts, resulting in remodelling of minichromosomes; the decline of HBV replication may be associated with the early decrease in histone modifications. HBx modulates not only the status of acetylation but also the methylation and phosphorylation of histone H3 bound to the cccDNA during HBV replication. Therefore, one of the mechanisms of HBx-dependent HBV replication may be that HBx influences epigenetic modifications, leading to chromatin remodelling.

Histone lysine methylation is closely related to gene transcription, which has a different effect at different amino-terminal residues. Methylation of lysines 9 and 27 in histone H3 correlates with gene repression, whereas methylation of lysines 4, 36 and 79 in histone H3 correlates with activation (27,28). Our study suggested that HBx modulates the status of hisone H3 methylation. The possible explanation from some studies is that SET and MYND domain-containing protein 3 (SMYD3) is one of the methyltransferases for H3 lysine 4 (29,30). RNA polymerase II is recruited to the promoter region of SMYD3 gene by HBx, leading to the enhancement of the expression of SMYD3 and cellular activities of HMTs at H3 lysine 4 (30,31). Histone modifications, as well as modifications of the DNA, can influence chromatin structure, induce the remodelling of chromatin and consequently result in gene silencing (27). HBx can increase the activities of total DNA methyltransferases (DNMTs) by upregulation of DNMT1, DNMT3A1 and DNMT3A2, and can selectively promote regional hypermethylation of specific tumour-suppressor genes (32). In the cytoplasm, HBx increases DNMT1 by activating the Ras signalling pathway (32,33) and/or by inhibiting p53 function (32,34). HBx interacts with DNMT3A to trigger epigenetic modifications at different loci, thus regulating the transcription of target genes. For example, HBx recruits DNMT3A to the promoter region of ML1F and IL4R, inducing inhibition of ML1F and IL4R and regional hypermethylation. In contrast, HBx separates DNMT3A from the promoter of IGFBP-3 and CDH6, resulting in activation of IGFBP-3 and CDH6 and downregulation of DNA methylation (35).

Histone H3 serine 10 phosphorylation is important for transcriptional activation and chromosome condensation occurring during mitosis, meiosis, apoptosis and DNA damage (27). Our study has shown that HBx modulates the status of histone H3 phosphorylation. HBx can also promote nuclear protein serine phosphorylation and increases pgRNA encapsidation and HBV DNA synthesis, which may be attributable to the activation of the HBx-induced signal transduction pathways including the core protein serine kinases (36). A rapid phosphorylation of histone H3 at serine 10 is induced by the early response genes c-fos and c-myc when the Ras mitogen-activated protein kinase (MAPK) signalling pathway is stimulated (37,38). Modifications of histones are quite complex and can interplay with each other due to the cross-talk between them (39). The activation of Aurora-kinase-B-mediated phosphorylation of H3 serine 10 may serve as a 'phos-methyl switch', leading to the separation of HP1 from heterochromatin while maintaining H3K9 methylation during mitosis (39,40). Acetylation of histones at lysines 9 and 14 can serve as a prelude to transcriptional activation, whereas methylation of histones at lysine 9 can lead to gene silencing and formation of heterochromatin. These modifications may influence the ability of serine 10 to be phosphorylated and vice versa (41).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are responsible for the steady-state balance of acetylation modification of histones, although this balance can be affected by HBx protein. Cellular histone acetytransferases p300, CBP, PCAF/GCN5, and the histone deactylases HDAC1 and hSirtl are recruited with different kinetics onto cccDNA in HBV replication (13,21). A previous study demonstrated that there is no HBx recruited onto cccDNA in cells replicating the HBx mutant HBV virus (21). cccDNA-bound histones are rapidly hypoacetylated in the absence of HBx, and the recruitment of p300 is severely impaired while the recruitment of the histone deacetylases HDAC1 and hSirtl is increased and occurs earlier (21). Our study also demonstrated that HBx modulates the status of histone H3 acetylation. The possible explanation is that HBx affects the expression of important cellular genes resulting from its transcriptional transactivation and transrepression properties. HBx can directly interact with the acetyltransferase p300/CBP complex in coordination to enhance the activity of CREB to promote transcription, leading to activation of the acetylated histone state of the target cellular genes (18,42). HBx recruits HDAC1 to the promoter of IGFBP-3 and induces the formation of the Sp1/HDAC1 complex, resulting in deacetylation of Sp1 and inhibition of the transcription of IGFBP-3 (43).

In summary, HBx plays a pivotal role in HBV replication and transcription. Specifically, HBx affects not only the status of acetylation but also methylation and phosphorylation of cccDNA-bound histone H3 during HBV replication in HepG2 cells. HBx may modulate HBV replication through the pathway of minichromosome remodelling related to HBV

replication. Further study is required to ascertain whether HBx modulates HBV replication by affecting the recruitment of other histone-modifying enzymes bound to the cccDNA in addition to HATs and HDACs. In this regard, our research provides experimental evidence for elucidating the mechanism of HBx-dependent HBV replication through the pathway of chromatin remodelling and identified the HBx protein as a new target for antiviral treatment at the level of cccDNA.

Acknowledgements

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