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Role of hepatitis B core protein in HBV transcription and recruitment of histone acetyltransferases to cccDNA minichromosome

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ABSTRACT

The hepatitis B core protein (HBc) has been suggested to interact with covalently closed circular DNA (cccDNA) and regulate hepatitis B virus (HBV) transcription. However, direct evidence is lacking. We aimed to identify the specific HBc region(s) responsible for transcription regulation and its interaction with cccDNA. Seventeen mutants with mutations at the four arginine-rich clusters of the HBc carboxyl-terminal domain (CTD) were created. The effect of HBc mutations on the levels of HBV DNA, RNA, and hepatitis B surface antigen (HBsAg) were measured. The association of cccDNA with mutant HBc and histone acetyltransferases (HATs) was assessed by chromatin immunoprecipitation (ChIP). Compared with wild-type HBc, HBc mutants with mutations in clusters III and IV resulted in a significant reduction in HBV RNA levels (all $P < 0.05$). HBc arginine clusters III and IV mutants also had a significantly lower levels of intracellular HBV DNA (<5% of wild-type; $P < 0.001$) and HBsAg (<10% of wild-type; $P < 0.0001$). cccDNA-ChIP assay demonstrated that HBc clusters III and IV mutants had a smaller degree of association with cccDNA ($P < 0.001$). In the HBc mutants, the association between HATs with cccDNA were reduced. In conclusion, HBc-CTD arginine residues at clusters III and IV play an important role in the regulation of HBV transcription as well as subsequent replication steps, likely through the reduced interaction of HBc with cccDNA and reduced acetylation of cccDNA-bound histones. These findings may provide clues to the identification of novel therapeutic targets against HBV.

1. Introduction

Currently approved therapeutic agents against chronic hepatitis B, despite very effective in the suppression of hepatitis B virus (HBV) replication, fail to completely eradicate HBV. This is partly due to the persistence of HBV covalently closed circular DNA (cccDNA) inside the infected hepatocytes. cccDNA is the template for the transcription of all HBV RNAs, and is not a direct target of current therapeutic agents. A better understanding of the HBV replication cycle, especially in HBV transcriptional regulation, is desirable for the development of novel effective therapy.

The hepatitis B core protein (HBc) plays multiple roles in HBV replication. HBc is a 21.5 kDa protein and is the structural component of the viral nucleocapsids. It contains a globular N-terminal domain and a C-terminal domain (CTD). The HBc N-terminal domain is essential for nucleocapsid formation. The HBc-CTD is arginine-rich, and the arginine residues can be grouped into four clusters named clusters I - IV, with each clusters being composed of 3 or 4 arginine residues.

During HBV replication, the HBV pregenomic RNA (pgRNA) is

Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; HBc, hepatitis B core protein; CTD, C-terminal domain; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA; ChIP, chromatin immunoprecipitation; CREB, cyclic AMP-responsive enhancer binding protein; CBP, CREB-binding protein; NTCP, sodium taurocholate cotransporting polypeptide; HBsAg, hepatitis B surface antigen; PCAF, P300/CBP-associated proteins; HATs, histone acetyltransferases.

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transcribed from cccDNA. The pgRNA is then encapsidated by HBc in the cytoplasm. Inside the viral capsids, HBc facilitates reverse transcription of the pgRNA to synthesize the HBV DNA minus and plus strands. This is attributed to the nucleic acid-binding properties of the HBc CTD (Lewellyn and Loeb, 2011; Nassal, 1992; Seeger and Mason, 2000). In addition to its action inside the nucleocapsids, HBc may also play a role in HBV replication inside the hepatocyte nucleus. Electron-microscopic and chromatin immunoprecipitation (ChIP) studies demonstrate that HBc is a component of the cccDNA minichromosome and involved in the epigenetic regulation of HBV transcription (Bock et al., 2001; Pollicino et al., 2006). HBc binds to cccDNA and reduces the nucleosome spacing of the cccDNA-histones complex, which may regulate HBV transcription by altering the nucleosomal arrangement of the HBV genome (Bock et al., 2001; Pollicino et al., 2006). The role of HBc in the regulation of HBV transcription is further reinforced by a study demonstrating that HBc preferentially binds to the CpG islands in cccDNA (Guo et al., 2011). The binding of HBc to cccDNA is associated with hypomethylation in CpG island 2 in cccDNA, increased binding of cyclic AMP-responsive enhancer binding protein (CREB) binding protein (CBP), and increased histone acetylation status, all of which are suggested to increase HBV transcription (Guo et al., 2011; Pollicino et al., 2006). It can be envisaged that blocking HBc-to-cccDNA binding can be a potential novel therapeutic approach. However, due to the lack of a convenient *in vitro* system that supports cccDNA-initiated HBV transcription, studies on the effect of HBc mutations on HBV transcription are limited.

The association between HBc and cccDNA is likely to be mediated by HBc arginine residues, which confer its nucleic acid binding property. In this study, we used a transient transfection system in which transcription of HBV RNA was originated from cccDNA (Pollicino et al., 2006) to assess the effects of HBc-CTD mutations on HBV replication. We aimed to identify the HBc arginine region(s) or residue(s) that are crucial to HBV transcription and replication. In addition, we aimed to study the interaction between mutant HBc and cccDNA using ChIP experiments. The relationship between HBc-cccDNA binding and HBV transcription and replication was also studied.

2. Materials and methods

2.1. Cell lines and plasmids

HepG2 cell expressing the sodium taurocholate cotransporting polypeptide (NTCP) was a kind gift from Professor DY Jin. Full-length HBV DNA was amplified from a 31 years old, hepatitis B e antigen positive, male chronic hepatitis B patient using primers and methods described previously (Gunther et al., 1995). The amplified HBV DNA was cloned into pUC19. Sequence analysis of the resulting clone, named pHBV3, revealed that it was of HBV genotype C, without any mutations in the basal core promoter and precore regions and drug resistance mutations. From pHBV3, a linear monomeric HBV DNA was released by digestion with the restriction enzyme *Sap*I, subjected to T4 ligase-mediated re-ligation, and used in subsequent transfection experiments. Mutagenesis of the HBc gene was performed using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Using pHBV3 as a template, an HBc-null HBV mutant, named HBcStop38 was created by introducing a nonsense mutation at codon 38 of the core gene, making an early truncated HBc protein (Pairan and Bruss, 2009).

A set of expression plasmids containing 17 HBc-CTD mutants, named MT1 to MT17, possessing mutations either at or in close proximity to the HBc-CTD arginine residues was created by site-directed mutagenesis. Sequences of the primers used for site-directed mutagenesis are tabulated in Supplementary Table 1. Mutations were confirmed by Sanger sequencing of the resulting plasmids (Supplementary Table 2). A schematic representation of the HBc mutants and their mutated regions is shown in Fig. 1. These mutants were grouped into four arginine clusters (cluster I: MT1-3; cluster II: MT4-6; cluster III: MT7-11; and cluster IV: MT12-17). Expression plasmids containing HBc were constructed by PCR amplification of the wild-type or mutated HBc gene using primers HBc-*Kpn*Is (5′-CTTGGGTACCTTTGGGGC*ATGGACAT*-3′; where the restriction site is underlined and the HBV sequence is italicized; same as below) and HBc-*Eco*RIa (5′-CAAGGAATTC*TAACATTGA-GATTCCC*-3′), followed by cloning of the *Kpn*I and *Eco*RI-digested PCR fragment into *Kpn*I and *Eco*RI-digested expression vector pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA).

2.2. Transient transfection

In all transfection experiments, full-length circularized HBV DNA was co-transfected into 10^6 HepG2-NTCP cells in a 6-well plate using the Lipofectamine 3000 reagent (Thermo Fisher Scientific), according to manufacturer's protocol, with or without co-transfection with the HBc expression plasmids. Transfection efficiency was monitored by the inclusion of 250 ng of a green fluorescent protein-expressing plasmid. A total of 1 μg DNA was used in each transfection. Unless otherwise stated, cells were harvested at 48 h post-transfection for subsequent analyses.

2.3. Extraction of HBV DNA and RNA

HBV DNA was purified from both intracellular core capsid particles and nuclei from transfected cells. Following centrifugation at 200×g at 4 °C for 15 min to collect the cell pellet, ice-cold homogenization buffer (50 mmol Tris-Cl pH 7.4, 1 mmol EDTA, and 1% Nonidet P-40) was added into the cell pellets for fractionation and lysis. The non-denaturing detergent nonidet P-40 can differentially break the plasma membrane without disrupting the nuclear membrane. The nucleic (pellet) and cytoplasmic (supernatant) fractions were then separated by centrifugation at 16,000×g at 4 °C for 15 min. Prior to the extraction of the encapsidated HBV DNA in the cytoplasmic fraction, 250 units of the Pierce Universal Nuclease (Thermo Fisher Scientific) were used to eliminate the excess residual input HBV DNA. Nucleic acids from both the nuclei pellet and cytoplasmic supernatant were extracted using the Purelink Genomic DNA mini kit (Thermo Fisher Scientific). The presence of transfected circularized HBV DNA in the nucleus was confirmed by specific detection of cccDNA by real-time PCR (methods described below in section 2.5). In the cytoplasm, only a negligible amount of circularized DNA (4.8 copies/μL of DNA extract; 0.0001% of the 8.2×10^6 copies/μL total HBV DNA) was amplified (Supplementary Fig. 1).

Total cellular RNA was extracted form transfected cells using TRIzol reagent (Thermo Fisher Scientific). Extracted RNA was treated with DNase I (New England Biolabs, Ipswich, MA) to prevent DNA contamination. DNA and RNA quantity and quality was estimated by measurement of A_{260} and A_{280} by the Nanodrop 2000 Spectrometer (Thermo Fisher Scientific).

2.4. Northern blot analysis of HBV RNA

For RNA analysis, 5 μg of RNA was resolved in 1% agarose gel containing 5% formaldehyde and transferred onto a Hybond-N membrane (GE Healthcare Life Sciences, Chicago, IL). The membrane was probed with DIG-labelled (Roche Life Science, Indianapolis, IN) full-length HBV DNA probes. Hybridization signals were detected by immuno-tagging with alkaline phosphatase conjugated anti-DIG antibody, followed by chromogenic detection using