Research Article

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Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection

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Background & Aims: The molecular biology of hepatitis B virus (HBV) has been extensively studied but the exact role of the hepatitis B X protein (HBx) in the context of natural HBV infections remains unknown.

Methods: Primary human hepatocytes and differentiated HepaRG cells allowing conditional trans complementation of HBx were infected with wild type (HBV(wt)) or HBx deficient (HBV(x−)) HBV particles and establishment of HBV replication was followed.

Results: We observed that cells inoculated with HBx-deficient HBV particles (HBV(x−)) did not lead to productive HBV infection contrary to cells inoculated with wild type HBV particles (HBV(wt)). Although equal amounts of nuclear covalently closed circular HBV-DNA (cccDNA) demonstrated comparable uptake and nuclear import, active transcription was only observed from HBV(wt) genomes. Trans-complementation of HBx was able to rescue transcription from the HBV(x−) genome and led to antigen and virion secretion, even weeks after infection. Constant expression of HBx was necessary to maintain HBV antigen expression and replication. Finally, we demonstrated that HBx is not packaged into virions during assembly but is expressed after infection within the new host cell to allow epigenetic control of HBV transcription from cccDNA.

Conclusions: Our results demonstrate that HBx is required to initiate and maintain HBV replication and highlight HBx as the key regulator during the natural infection process.

Keywords: Hepatitis B virus; HBx protein; Natural infection; Primary human hepatocytes; HepaRG.

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Abbreviations: HBV, hepatitis B virus; HBx, hepatitis B virus X protein; PHH, primary human hepatocytes; cccDNA, covalently closed circular DNA; ChIP, chromatin immunoprecipitation.

Introduction

HBV is a small, enveloped DNA virus which replicates its genome via an RNA intermediate. The encapsidated viral genome consists of a 3.2 kb partially double stranded relaxed circular DNA (rcDNA) molecule. Upon translocation to the nucleus, the rc genome is converted into cccDNA which serves as template for viral transcription. The 3.5 kb pre-genomic RNA (pgRNA) is the mRNA for the synthesis of polymerase and core proteins but also the template for the reverse transcription. The 2.4/2.1 kb subgenomic RNAs encode for three viral envelope proteins. In the cytoplasm, pgRNA is encapsidated and reverse transcribed within the viral capsid into the rcDNA genome. Mature viral capsids are then either directed to the secretory pathway for envelopment or redirected toward the nucleus to establish a cccDNA pool.

Beside structural proteins, HBV encodes for two non-structural proteins – HBe and HBx – of less defined functions. The secreted HBe (also named HBeAg) is supposed to be immunoregulatory [8], whereas HBx seems to interact with various cellular partners and modify diverse cellular processes [2]. Its exact role in the viral life cycle, however, has not been defined yet.

It has been shown in the woodchuck model of HBV infection that woodchuck hepatitis B virus (WHV) X protein is needed to establish productive infection in the animal [7,38]. Low level replication *in vivo* allowed genotypic reversions to wild type WHV [37] pointing at the importance of WHV X. The importance of the human HBx in the context of HBV infection was demonstrated recently using human hepatocyte chimeric mice. HBx deficient HBV developed measurable viremia only in HBx-expressing livers [31]. In addition, it was shown that HBx-deficient HBV genomes are strongly attenuated for HBV replication using HBV transgenic mice [35], hydrodynamically-injected mice [15,16], or cell culture models [1,3,15,18]. Whereas HBx-deficiency had little effect on HBV replication in human hepatoma Huh7 cells, replication was impaired in HepG2 cells [3,15,16,18].

Different functions have been attributed to HBx regarding HBV replication. Several studies indicated that HBx stimulates HBV replication by activating viral transcription [18,30,36,37] and enhancing viral polymerase activity via calcium signaling pathways [5,6,17]. HBx may also enhance pgRNA encapsidation

by increasing phosphorylation of the viral core protein [20]. In primary rat hepatocytes, HBx seems to regulate cell cycle progression and thereby stimulate HBV replication [10]. Although these studies suggested pleiotropic functions of HBx using transfection or transduction of HBV genomes, none of them assessed the role for HBx in the setting of a complete infection cycle. Moreover, contradictory results obtained in surrogate models highlight the importance of performing experiments in the context of an HBV infection that allows studying the complete life cycle.

For years, freshly prepared primary human hepatocytes (PHH) were the only cells known to support HBV infection [11]. However, the access to human livers is restricted and the quality of cells varies. More recently, the discovery of the HepaRG cell line, which supports HBV infection and replication, has opened new perspectives. Specificity of *in vitro* HBV infection has been demonstrated using neutralizing antibodies and HBV envelope-derived peptides [12,13]. HepaRG cells are bi-potent liver progenitor cells that may be differentiated into both biliary epithelial- and hepatocyte-like cells [21]. HepaRG cells are capable of long-term stable expression of liver-specific metabolizing enzymes and membrane transporters – both indicators of highly differentiated hepatocytes closely correlating with susceptibility to HBV infection [12,25].

Using the two relevant *in vitro* models of HBV infection, we showed here that HBx is essential for productive HBV infection when the natural HBV transcription template – cccDNA – has been established.

Materials and methods

Ethics statement

Primary human hepatocytes (PHH) were isolated from surgical liver specimens obtained during partial hepatectomy. Informed consent was obtained from each patient, and the procedure was approved by the local Ethics Committee.

HBV inocula, cell cultures, and HBV infection

HBV particles were concentrated from the supernatant of HepG2.2.15 (HBV(wt1)), HepG2 H1.3 (HBV(wt2)), or HepG2 H1.3Δx (HBV(x−)) cells cultivated in Williams E medium containing 5% fetal calf serum and 1% DMSO using centrifugal filter devices (Centricon Plus-70, Biomax 100.000, Millipore Corp., Bedford, MA). HepG2 H1.3Δx cells were established by stable integration of a 1.3-fold HBV genome (genotype D, subtype ayw) carrying premature stop codon mutations in both the 5′ and 3′ HBx open reading frames [32]. HepG2 H1.3Δx/x+ cells were generated by transduction of HepG2 H1.3Δx cells with a lentivirus containing the HBx transgene (genotype D, serotype ayw). HBV inocula were titered by HBV-DNA dot blot analysis after sedimentation into a CsCl density-gradient. Only enveloped DNA-containing viral particles (vp) were taken into account when the multiplicity of infection was calculated [14,25]. Only inocula reaching a titer between 3×10^9 and 3×10^{10} vp/ml were used for infection assay in order to keep the inoculum <10% of the total medium volume. PHH were isolated from the surgically-removed liver sections, cultured, and infected with HBV as described previously [27]. HepaRG cell culture, differentiation, and HBV infection have also been described [12,13].

Transcomplementation of HBx

HepaRG-TR cell line stably over-expressing the tetracycline repressor (TR) was obtained after high-titer transduction with a lentivirus produced from pLenti6-TR plasmid (Invitrogen, Carlsbad, USA). Blasticidin-resistant HepaRG-TR cells were then transduced with a second lentivirus conferring zeocin resistance and containing an HBx (genotype D, serotype ayw, Galibert's strain) transgene cloned into pLenti4/TO plasmid under the control of a tetracycline-regulated promoter. In addition, constitutively HBx-expressing lentivirus was established and used to express HBx in HepG2 H1.3Δx and PHH. To confirm HBx expression, Western blot analyses were performed using an anti-HBx antibody from Affinity Bioreagent (MAI-081, Golden, CO, USA).

Analysis of HBV replication intermediates

HBeAg and HBsAg were determined using commercial immunoassays (Siemens Molecular Diagnostics, Marburg and Abbott Laboratories, Wiesbaden, Germany). Total DNA was purified from infected cells using DNA MiniElute Kit (Qiagen, Hilden, Germany). Total RNA was extracted from infected cells and transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA). HBV-DNA, pgRNA, and cccDNA were detected using specific PCR primers [24,33]. HBV-DNA was quantified relative to an external plasmid standard. cccDNA is expressed as normalized ratio to mitochondrial DNA, pgRNA as normalized ratio to GAPDH [24,33] as previously described [25]. Real-time PCRs (qPCR) were performed using the LightCycler™ system (a dilution series of a calibrator was included in each PCR run) and analyzed using the second derivative maximum method that includes both normalization to the reference gene (mitochondrial DNA for cccDNA and *GAPDH* for pgRNA, *HNF4a* and *TDO*) and to primer efficiency (Roche Diagnostics, Mannheim, Germany) [14,25].

Chromatin immunoprecipitation (ChIP) assays

Cells were harvested and incubated in lysis buffer (Pipes 5 mM pH 8, KCl 85 mM, NP40 0.5%) containing protease inhibitors (PIC from Sigma–Aldrich, St. Louis, MO, USA) for 10 min at 4 °C. After centrifugation, nuclei were fixed in 1% formaldehyde for 20 min at 4 °C, cross-linked and extracted with a SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, PIC). Chromatin solutions were sonicated to generate 300- to 400-bp DNA fragments. After centrifugation, supernatants were diluted 1:10 (in 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl containing PIC) and pre-cleared with blocked protein G-Plus (Thermo Fisher Scientific, Rockford, IL, USA). ChIP was then performed for 14–16 h at 4 °C using antibodies specific to AcH3 (rabbit polyclonal IgG recognizing histone H3 di-acetylated at lysine 9 and 14; Upstate Biotechnology, Millipore, Billerica, MA, USA) and nonspecific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a negative control. Immunoprecipitated chromatins were processed and analyzed by PCR and real-time PCR using cccDNA selective primers and probes as described before [1].

Results

HBx is not required for efficient virion production by hepatoma cells with an integrated HBV genome

HBV virions were generated from stable HBV-producing human hepatoma cell lines: HBV(wt) particles were obtained from HepG2.2.15 [28] and HepG2 H1.3 [14,24]. HBV(x−) virions were produced by the HepG2 H1.3Δx cell line that replicates HBV from a linearized 1.3-fold over-length genome and also carries a premature stop codon at amino acid position 7 after the ATG in both HBx open reading frames [36]. Analysis of concentrated supernatant from the different cell lines showed that enveloped HBV particles were released from HepG2.2.15 or HepG2 H1.3 as well as from HepG2 H1.3Δx cells (Supplementary Fig. 1), suggesting that HBx is not essential for HBV transcription and replication from an integrated linear HBV DNA transgene and that assembly can take place in the absence of HBx protein in HepG2 cells.

Of note, HBV(wt) and HBV(x−) virions could also be purified from HepG2 cells transduced with either recombinant baculoviruses or adenoviruses carrying the corresponding HBV genome [19,33], or from HepG2 cells transfected with plasmids encoding HBV (data not shown). Infectivities of resulting HBV(wt) inocula were comparable whether HBV Dane particles were purified or not (Supplementary Fig. 2). Thus the following infection experiments were performed with HBV particles concentrated using centrifugal filter devices.