

Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function

Laura Belloni^{a,b,1}, Teresa Pollicino^c, Francesca De Nicola^{d,e}, Francesca Guerrieri^{a,e}, Giuseppina Raffa^c, Maurizio Fanciulli^{d,e}, Giovanni Raimondo^c, and Massimo Levrero^{a,b,e,1}

^aLaboratory of Gene Expression, Fondazione A. Cesalpino, 00161 Rome, Italy; ^bDepartment of Internal Medicine, University La Sapienza, 00185 Rome, Italy; ^cDepartment of Internal Medicine, University of Messina, 98122 Messina, Italy; ^dDepartment of Molecular Oncogenesis, Centro della Ricerca Sperimentale, 00158 Rome, Italy; and ^eRome Oncogenomic Center, Istituto Regina Elena, 00161 Rome, Italy

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HBV cccDNA, the template for transcription of all viral mRNAs, accumulates in the nucleus of infected cells as a stable episome organized into minichromosomes by histones and non-histone viral and cellular proteins. Using a cccDNA-specific chromatin immunoprecipitation (ChIP)-based quantitative assay, we have previously shown that transcription of the HBV minichromosome is regulated by epigenetic changes of cccDNA-bound histones and that modulation of the acetylation status of cccDNA-bound H3/H4 histones impacts on HBV replication. We now show that the cellular histone acetyltransferases CBP, p300, and PCAF/GCN5, and the histone deacetylases HDAC1 and hSirt1 are all recruited *in vivo* onto the cccDNA. We also found that the HBx regulatory protein produced in HBV replicating cells is recruited onto the cccDNA minichromosome, and the kinetics of HBx recruitment on the cccDNA parallels the HBV replication. As expected, an HBV mutant that does not express HBx is impaired in its replication, and exogenously expressed HBx transcomplements the replication defects. p300 recruitment is severely impaired, and cccDNA-bound histones are rapidly hypoacetylated in cells replicating the HBx mutant, whereas the recruitment of the histone deacetylases hSirt1 and HDAC1 is increased and occurs at earlier times. Finally, HBx mutant cccDNA transcribes significantly less pgRNA. Altogether our results further support the existence of a complex network of epigenetic events that influence cccDNA function and HBV replication and identify an epigenetic mechanism (i.e., to prevent cccDNA deacetylation) by which HBx controls HBV replication.

histone acetylation | HATs | HDACs

Hepatitis B virus (HBV) infection is a major health problem, with ≈ 400 million people chronically infected worldwide who are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) (1). The epidemiological evidence linking HBV infection to HCC is very strong, and despite the mechanisms underlying HBV-associated carcinogenesis remain to be fully defined, a growing number of studies support a direct role of HBV in the process (2–5). The HBV-encoded regulatory protein hepatitis B virus X protein (HBx) is thought to contribute to HBV oncogenicity (5, 6). HBx transforms SV40-immortalized murine hepatocytes, induces cell cycle progression within the regenerating liver, causes liver cancer in some transgenic mice, and acts as a cofactor to accelerate cancer development in other mouse models (6–11). HBx is a 154-amino acid protein with an N-terminal negative regulatory domain and C-terminal transactivation or coactivation domain that has been detected both in the cytoplasm and in the nuclei of infected hepatocytes (6, 12, 13). Studies in transfected cells have shown that HBx expression affects several cellular functions such as cytoplasmic calcium regulation, cell signaling, transcription, cell proliferation, DNA repair, and apoptosis (11, 13–16). To perform its multiple functions, HBx interacts with many cellular partners including the tumor suppressor p53, the UV-stimulated E3-ubiquitin ligase DDB1, the nuclear export protein CRM1, a number of nuclear proteins involved in the regulation of tran-

scription such as the RPB5 subunit of RNA polymerase II, TFIIB, TFIIF, the TATA binding protein (TBP), the basic domain-leucine zipper (bZIP) family transcription factors ATF2, CHOP, and cAMP-response element (CRE)-binding protein (CREB) (6, 14). Recently, it has been shown that HBx interacts and cooperate with the CREB-binding protein (CBP)/p300 to modify chromatin dynamics of target genes and to synergistically enhance CREB activity (17).

The lack of homology of the X ORF to host protein and its high conservation to other mammalian hepadnaviruses genomes strongly suggest that HBx play a role in viral life cycle (6). Although initial studies suggested that HBx was not required for virus replication in cell culture (18), experiments with the highly related woodchuck hepatitis virus (WHV) system indicate that the WHV X protein (WHx) is required for virus replication *in vivo* (19–22). Studies performed using a plasmid-based replication assays that use greater-than-unit-length HBV genomes transfected in HCC cell lines or injected via the mouse tail vein under hydrodynamic conditions have repeatedly confirmed that HBx potentiate HBV replication (23–27).

The HBV replicative intermediate cccDNA (covalently closed circular HBV DNA), which serves as a template for the transcription of all viral transcripts including the pregenomic RNA (pgRNA), is organized into a minichromosome in the nuclei of infected hepatocytes by histone and non-histone proteins (28). The HBV core protein is a structural component of the HBV minichromosome, and its binding results in a reduction of the nucleosomal spacing of the HBV nucleoprotein complexes (29). Recently, we have developed a ChIP-based quantitative technique to study the recruitment *in vivo* of cellular and viral proteins onto the HBV minichromosome (30). Chromatin immunoprecipitation (ChIP) techniques allow the identification *in vivo* of the DNA binding sites of virtually any chromosome component (31). The principle relies upon the fixation of protein-DNA and protein-protein interactions *in vivo* by the cross-linking agent formaldehyde, and the immunoprecipitation of the cross-linked protein of interest with specific antibody directed against immunoprecipitated DNA is used as a template in real-time PCR. The HBV cccDNA ChIP assay combines a cccDNA ChIP step with a sensitive and specific real-time PCR protocol for cccDNA quantification (30). Using an anti-acetylated-H3 or -H4 cccDNA ChIP assay, we found that HBV

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¹To whom correspondence may be addressed. E-mail: laura.belloni@gmail.com or massimo.levrero@uniroma1.it.

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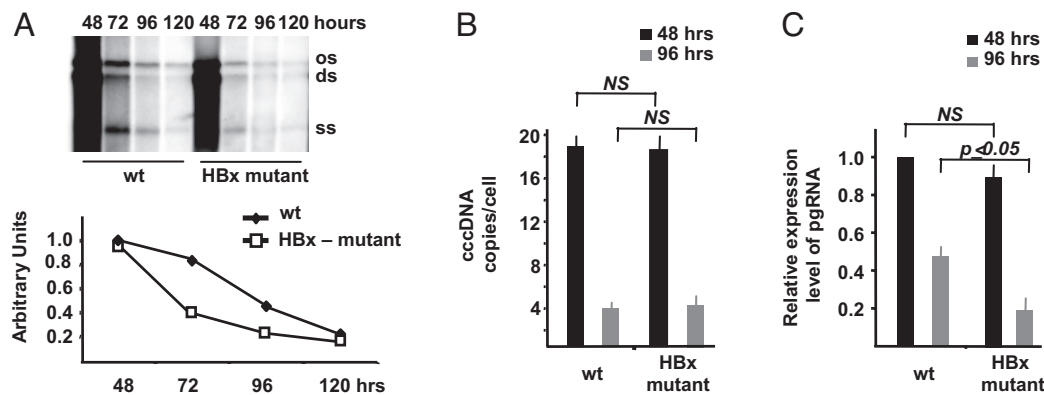


Fig. 1. HBx is involved in viral replication, and it is required for HBV pregenomic RNA transcription. (A) HepG2 cells were transfected with monomeric linear full-length WT or HBx mutant HBV adw genomes, and cytoplasmic HBV core particles were isolated at the indicated time points after transfection. (Upper) Southern blot analysis of HBV DNA replicative intermediates. (Lower) Densitometric quantification of HBV replicative intermediates. Signal intensity of the single-strand (SS) band underneath the linear double-stranded (DS) HBV DNA band was quantified with Quantity One 1-D Analysis Software (BioRad Laboratories). The band corresponding to the D5 HBV DNA was not included in the quantitative analysis, because this DNA may be partially derived from transfected input DNA. The optical density value of the SS band at 48 h in WT replicating cells is set at 1.0. Data are expressed as relative arbitrary units (mean + SD) from three independent experiments. OC, open circular duplex HBV DNA; DS, double-strand; and SS, single-strand HBV DNA replicative intermediates. (B) cccDNA accumulation in HepG2 cells transfected with WT or HBx mutant HBV genomes. Real-time quantitative PCR analysis was performed using selective cccDNA primers to amplify cccDNA and beta-globin primers to normalize the DNA samples. Results are expressed as number of cccDNA copies per cell (mean + SD) from four independent experiments. (C) HepG2 were transfected with WT or with HBx mutant HBV genomes and mRNAs were extracted 48 and 96 h posttransfection as described in the *Materials and Methods* section. Specific primers were used to quantitate the HBV pregenomic RNA, and GAPDH amplification was used to normalize for equal loading of each RNA sample. Results are expressed as number of cccDNA copies per cell (mean + SD) from three independent experiments. pgRNA, HBV pregenomic RNA.

replication is regulated, both in HBV replicating Huh7 cells and in the liver of HBV chronically infected patients by the acetylation status of H3/H4 histones bound to the viral cccDNA (30). Here we investigated the recruitment of HBx onto the HBV minichromosome and its ability to regulate the epigenetic control of cccDNA function in HBV replicating cells.

Results

HBx Potentiates HBV Replication. The HBV regulatory protein HBx has been shown to potentiate viral replication in plasmid-based replication assays that use greater-than-unit-length HBV genomes transfected in HCC cell lines or injected via the mouse tail vein under hydrodynamic conditions (23–27). We have recently shown that transfection of plasmid free linear HBV DNA into Huh7 and HepG2 HCC cell lines (32) allows to fully recapitulate the HBV replication cycle, including the nuclear generation of viable cccDNA (30) (Fig. S1). To examine the contribution of HBx to viral replication in our cccDNA-driven replication system, equivalent numbers of HepG2 cells were transfected with either linear wild-type (WT) HBV genomes or with a mutant HBV DNA carrying a stop codon for amino acid 8 of the HBx ORF. Cells were harvested at the indicated times posttransfection, cytoplasmic viral core particles were isolated, and HBV DNA was extracted, analyzed by hybridization with a ^{32}P -labeled full-length HBV DNA probe, and quantitated by densitometric analysis. As shown in Fig. 1A, the level of capsid associated HBV DNA in cells transfected with the HBx mutant DNA was reduced as compared to the levels measured in cells transfected with WT HBV DNA. Whereas the peak replication at 48 h is only marginally affected by the lack of HBx, the HBx mutant virus displays a faster decrease in replication at 72 and 96 h posttransfection as compared to WT HBV. These results confirm that HBx potentiates HBV replication and suggest that HBx activity is required to sustain replication in our assay.

HBx Is Required for HBV Pregenomic RNA Transcription. To investigate the mechanism involved in HBx-induced potentiation of HBV DNA replication, we measured the size of the cccDNA

pool and the levels of pgRNA in HepG2 cells replicating either the WT or the HBx mutant HBV virus. The median number of cccDNA copies per hepatocyte (33) was similar in cells replicating WT or HBx mutant viruses both at 48 h (replication peak) and at 96 h posttransfection, when both cccDNA levels and HBV replication are sharply reduced (30) (Fig. 1B). On the opposite, the levels of pgRNA were slightly lower at 48 h and reduced by 50% to 70% at 96 h in HepG2 cells replicating the HBx mutant virus ($P \leq 0.05$) (Fig. 1C). Altogether, these results indicate that HBx is not required for cccDNA maturation but modulates pgRNA transcription from cccDNA.

The HBx Viral Protein Is Recruited onto the cccDNA In vivo. Nuclear cccDNA molecules are organized into a chromatin like structure by histone and non-histone proteins. Using the cccDNA ChIP assay, we have been able to confirm that the HBV core protein is recruited onto the viral chromatin (29, 30). HBx does not bind specific DNA sequences, but it is recruited onto the cellular chromatin through its ability to interact with cellular transcription factors and cofactors (17). To assess whether HBx interacts with the cccDNA to modulate its transcription, we have performed an anti-HBx cccDNA ChIP assay in HBV replicating HepG2 cells. We found that endogenous HBx binds to the cccDNA (Fig. 2A) at 48 h posttransfection. The kinetics of HBx recruitment onto the cccDNA was assessed in HepG2 cells cotransfected with WT linear HBV DNA and an HA-tagged HBx expression vector. Using an anti-HA antibody to perform the cccDNA ChIP assay, we found that the recruitment of exogenously expressed HA-tagged HBx onto the cccDNA closely parallels the kinetics of cccDNA bound H3 acetylation (Fig. 2B). Altogether, these results suggest that HBx might interact with the cccDNA to modulate its transcription.

Epigenetics Changes in HepG2 Cells Replicating HBx Mutant HBV. cccDNA transcriptional activity and HBV replication are modulated both in in vitro cellular systems and in vivo in the liver of chronic HBV carriers by epigenetic changes of cccDNA-bound H3 and H4 histones (30). Moreover, HBV replication in Huh7 cells

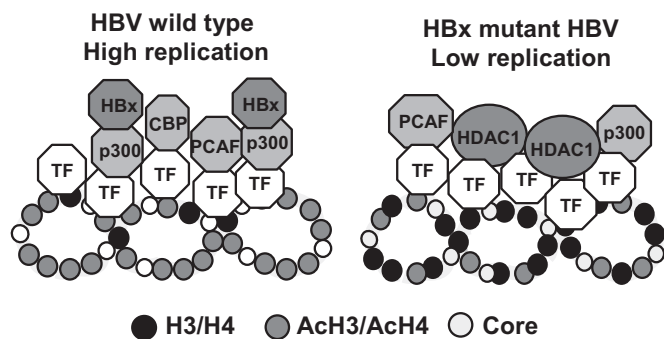


Fig. 4. Schematic representation of cccDNA-bound histones acetylation status and the recruitment of chromatin modifying enzymes onto the viral minichromosome in relation to viral replication and HBx status. In cells replicating an HBx mutant HBV, cccDNA-bound histones are hypoacetylated, the recruitment of the p300 acetyltransferase is severely impaired, whereas the recruitment of the histone deacetylases hSirt1 and HDAC1 is increased. This behavior is similar to what is observed in the later phase (96–144 h) of the *in vitro* viral replication cycle in our model system and mimicks the situation found in the liver of anti-HBe patients with low viremia (30).

plasm (12, 13). HBx has also been shown to modulate chromatin dynamics in HCC cells and tissues. HBx binds several nuclear proteins involved in the regulation of transcription, including components of the basal transcriptional machinery (RPB5, TFIIB, TBP, TFIID), coactivators (CBP, p300, and PCAF), and transcription factors (ATF/CREB, ATF3, c/EBP, NF-IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, p53) (14, 34). HBx is bound *in vivo* to the promoters of a number of CREB-regulated genes and favors transcription by increasing the amount of CBP and p300 recruited on the same promoters (17). In addition to stimulate transcription, HBx can also repress gene expression by upregulating DNMT1, DNMT3A1, and DNMT3A2 levels, increasing total DNA methyltransferase (DNMT) activity, and selectively facilitating the regional hypermethylation of the promoters of certain tumor suppressor genes (35, 36). More recently, it has been shown, by ChIP and sequential-ChIP experiments that HBx recruits DNMT3A on promoters of the repressed genes MT1F and IL4R leading to promoter hypermethylation and suppression of gene expression (37). In the presence of HBx, DNMT3A dissociates from the promoter region of the upregulated genes IGFBP3 and CDH6, thus resulting in hypomethylation and transcriptional activation (37).

Here, coupling cccDNA quantification (38) and the cccDNA ChIP assay (30), we show that the HBx produced in HBV replicating cells is recruited onto the cccDNA minichromosome. HBx recruitment on the cccDNA parallels HBV replication and the recruitment of the PCAF/GCN5, p300, and CBP acetyltransferases. We also found that cccDNA-bound histones are more rapidly hypoacetylated in cells replicating the HBx mutant, and the recruitment of the p300 acetyltransferase is severely impaired, whereas the recruitment of the histone deacetylases hSirt1 and HDAC1 is increased and occurs earlier (Fig. 4). Accordingly, we show that in cells replicating the HBx mutant, the pool of cccDNA in the nuclei is not reduced, but the HBx mutant cccDNA transcribes significantly less pgRNA. Although our observations imply that HBx exerts its role in potentiating HBV replication by acting directly on the cccDNA, we cannot exclude that Ca^{++} mobilization and src activation (13, 15) might also play a role. Importantly, it has been recently reported that HBx protein localized to the nucleus restores HBx-deficient virus replication in HepG2 cells and *in vivo* in hydrodynamically injected mice (39), thus supporting the importance of HBx interaction with the cccDNA in the nucleus. Altogether our results indicate that HBx influences the epigenetic control of nuclear HBV

cccDNA function by modulating the recruitment of chromatin-modifying enzymes onto the viral minichromosome.

Materials and Methods

Transient Transfection of Full-Length HBV DNA Genomes. Full-length WT and HBx mutant HBV genomes of genotype A (adw) were released by *EcoRI* (New England Biolabs) digestion from replication-competent plasmids HTD and HBX-21 HTD (18) (a gift from Hussein Sirma and Hans Will, Hamburg, Germany), containing complete WT and HBx mutant HBV DNA dimers, respectively. In particular, the pHBX-21 HTD carries a single nucleotide substitution that results in a termination site at the codon 8 of the X ORF. After digestion, the 3.2-kb fragments were recovered by gel purification using the QIAquick gel extraction kit (Qiagen) and inserted in the *EcoRI* site of pCR11-TOPO vector (Invitrogen) to generate the pCR.HBV.A.EcoRI and the pCR.mtHBx.A.EcoRI plasmids. Monomeric linear full-length WT and HBx HBV genomes (32) were released from the pCR.HBV.A.EcoRI and the pCR.mtHBx.A.EcoRI plasmids using *EcoRI-PvuII* (New England Biolabs) and transfected into HepG2 human hepatoma cells using the IT-LT1 reagent (Mirrus Biotrans). Briefly, HepG2 cells were seeded at a density of 2–3 millions of cells in 100-mm diameter Petri dishes and transfected 24 h later with 1–2 μ g digested HBV DNA. Unless otherwise specified, culture medium was changed 1 day after transfection, and cells were harvested at the indicated times posttransfection. All transfection included 0.5 μ g green fluorescence protein expression vector (GFP) to assess transfection efficiency (HepG2 cells range 28–32%).

Purification and Analysis of HBV DNA from Core Particles. HBV DNA was purified from intracellular core particles and examined by Southern blot analysis as described (33) (*SI Text*).

HBV cccDNA and pgRNA Quantification. Nuclear HBV cccDNA was isolated from HBV-transfected HepG2 cells and quantified by real-time PCR as described (33 and *SI Text*). Total RNA was extracted from HBV-transfected HepG2 cells using the TRizol reagent (Invitrogen). RNA samples were treated with RQ1 RNase-Free DNase (Promega) for 30 min at 37 °C and stored until used. RNA quality and quantity were monitored by ethidium-bromide staining and by UV absorbance. For pgRNA analysis, 2 μ g DNase-treated RNA were reverse-transcribed and amplified by the ThermoScript RT-PCR system (Invitrogen). Two microliters of each cDNA were quantitated by real-time PCR analysis (Light Cycler; Roche Diagnostics) using the following pgRNA specific primers and probes: Forward primer 5'-GCCTTAGAGTCTCTGAGCA-3', reverse primer 5'-GAGGGAGTCT TCTTCTAGG-3', FRET hybridization probes 5'-AGTGTG-GATTGCACTCTCCAGC FL-3', Red640-5'-ATAGACCACCAATGCCCTATCT-TATCAAC-3'. The h-G6PDH housekeeping gene Light Cycler set (Roche Diagnostics) was used to normalize the RNA samples.

cccDNA Chromatin Immunoprecipitation Assays. The cccDNA ChIP assays were performed as described, with minor modifications (30 and *SI Text*). Cross-linked chromatin was subjected to immunoprecipitation for 14–16 h at 4 °C using antibodies specific to H4, AcH4, AcH3, p300, PCAF, E2F1, hSirt1, HDAC1, HBV-HBx (listed in *SI Text*). Immunoprecipitations with relevant nonspecific immunoglobulins (Santa Cruz Biotechnology) were included in each experiment as a negative control. Immunoprecipitated chromatins were processed and analyzed by PCR and real-time PCR using cccDNA selective primers and probes that discriminate between cccDNA and the relaxed circular DNA (OC species) present in cytoplasmic HBV particles (30, 38).

Immunoblotting. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.6, 1% Nonidet P-40, 140 mM NaCl, 0.1% SDS), and the insoluble pellet was discarded after centrifugation. Protein concentration was determined by the BCA protein assay reagent (Bio-Rad Laboratories). Protein lysates were transferred to nitrocellulose membrane and incubated with HA antibodies from Santa Cruz Biotechnology (catalog no. sc-805). Autoradiography images were analyzed with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories), and signal intensity was quantitated using the Quantity One 1-D Analysis Software (Bio-Rad Laboratories).

Statistical Analysis. Continuous variables were expressed as median. Two group comparisons of continuous variables were performed using the nonparametric Wilcoxon rank sum test. *P* values < 0.05 were considered statistically significant.

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