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The C-terminal region of the hepatitis B virus X protein is essential for its stability and function

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Abstract

More than 350 million people worldwide are chronically infected with the human hepatitis B virus (HBV). Chronic HBV infections are associated with the development of hepatocellular carcinoma. While the mechanism of HBV-associated carcinoma remains undefined, it is thought to involve a combination of a continuous inflammatory response to HBV-infected hepatocytes and activities of HBV proteins such as the HBV X protein (HBx). HBx stimulates HBV replication; however, the mechanism by which HBx stimulates HBV replication remains incompletely understood. Studies performed with the woodchuck hepatitis virus (WHV) in woodchucks demonstrated that a C-terminally truncated mutant of the WHV X protein could not stimulate WHV replication. However, whether the C-terminus of HBx is important for HBx-stimulation of HBV replication is unclear. We have constructed C-terminal truncation mutants of HBx and have demonstrated that the C-terminus of HBx impacts HBx stability, HBx activation of transcription, and HBx stimulation of HBV replication. These observations highlight the impact of the HBx C-terminus on HBx activities and the importance of directly analyzing HBx expression and functions in HBV-associated tumors that contain chromosomal integrants of HBV with truncations of the HBx gene.

1. Introduction

Globally, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third highest cause of cancer-associated deaths (reviewed in (Block et al., 2003; McKusick et al., 1986)). It is estimated that at least 50% of HCC cases are caused by chronic infections of the liver with the human hepatitis B virus (HBV), a member of the Hepadnavirus family (reviewed in (Block et al., 2003; Seeger, 2007)). The mechanisms underlying the development of HBV-associated HCC are thought to involve a combination of continuous immune-mediated destruction of HBV-infected hepatocytes and resultant hepatocyte regeneration in individuals with a chronic HBV infection as well as activities of HBV proteins such as the HBV X protein (HBx) (reviewed in (Seeger, 2007)). The HBV genome

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Conflict of interest

None to declare.

is a highly compact, partially double-stranded, circular DNA that contains four overlapping, open reading frames encoding the HBV core, envelope, polymerase/reverse-transcriptase, and HBx proteins (reviewed in (Seeger, 2007)). The Hepadnavirus family consists of related viruses that infect various mammalian and avian species (reviewed in (Seeger, 2007)); only chronic infections with mammalian hepadnaviruses are associated with the development of HCC (reviewed in (Ganem, 1996)). Interestingly, only mammalian hepadnaviruses encode an X protein while avian hepadnaviruses either do not encode an X protein or encode a highly divergent X protein (Mandart et al., 1984; Sprengel et al., 1985). The results of numerous studies suggest that HBx stimulates HBV replication and can impact cell transformation processes in some cell culture and *in vivo* model systems ((Keasler et al., 2007; Kim et al., 1991; Lee et al., 1990; Melegari et al., 1998; Xu et al., 2002; Yu et al., 1999) and reviewed in (Bouchard and Schneider, 2004; Seeger, 2007). In contrast, studies investigating activities of the highly divergent duck hepatitis B virus (DHBV) X protein have shown that this protein is not required for DHBV replication (Chang et al., 2001; Meier et al., 2003). Overall, these studies emphasize the importance of understanding functions of HBx and how HBx activities impact HBV replication and hepatocyte physiology.

HBx is a 17.5 kDa, 154 amino acid, non-structural HBV protein that is localized to the nucleus, cytoplasm, and mitochondria of HBx-expressing cells ((Clippinger and Bouchard, 2008; Henkler et al., 2001; McClain et al., 2007) and reviewed in (Bouchard and Schneider, 2004)). The half-life of cytosolic, soluble HBx is approximately 15 minutes, and the half-life of cytoskeletal-associated HBx is about 3 hours (Schek et al., 1991). HBx is a multifunctional protein that can interact with various cellular factors and modulate cellular transcription, apoptosis, and proliferation pathways (reviewed in (Bouchard and Schneider, 2004)). HBx activates several transcription factors including nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) (Chirillo et al., 1996; Su and Schneider, 1996; Waris et al., 2001).

Many studies have analyzed the impact of X protein expression on replication of mammalian hepadnaviruses. For example, the results of two studies suggested that the woodchuck hepatitis virus (WHV) X protein (WHx) strongly enhances WHV replication in woodchucks and that WHV mutants which either do not produce WHx or contain C-terminal truncations of WHx replicate at lower levels than the wild-type virus (Chen et al., 1993; Zoulim et al., 1994). In contrast, the results of one study demonstrated that WHV that did not express WHx could replicate in woodchucks, albeit at very low levels; however, circulating WHV found in woodchucks that had been inoculated with this WHx mutant WHV had reverted to wild-type WHV (Zhang et al., 2001). Consequently, while the results of this study suggest that WHx may not be absolutely required for WHV replication, it none-the-less confirms that WHx expression strongly impacts WHV replication. In HBV-transgenic mice or mice hydrodynamically injected with a plasmid that contains a cDNA copy of the HBV genome, HBx stimulated HBV replication; the impact of HBx was most apparent in mice hydrodynamically injected with plasmids encoding the HBV genome as compared to injections with a mutated copy of the HBV genome that could not express HBx (Keasler et al., 2007; Xu et al., 2002; Yang et al., 2002). HBx-induced stimulation of HBV replication has also been studied in various cell lines. HBx-deficient HBV did not replicate well in HepG2 cells, a human hepatoblastoma cell line, but did replicate efficiently in Huh7 cells, a human hepatoma cell line (Blum et al., 1992; Melegari et al., 1998). The reasons for cell-line variations in HBx-dependent HBV replication are unclear. Cumulatively, the results of all these studies suggest that HBx has an important role during HBV replication. HBx stimulation of cellular calcium signaling pathways, regulation of the proteasome, interaction with DNA-damage binding protein 1 (DDB1), and regulation of the mitochondrial permeability transition pore (MPTP) have been linked to its regulation of HBV replication (Bouchard et al., 2001b; Leupin et al., 2005; Tan et al., 2009; Zhang et al., 2004).

The potential link between X protein expression in mammalian hepadnaviruses and the development of HCC has led several groups to use HBx-transgenic mice to analyze the role of HBx in the development of HBV-associated liver cancer ((Kim et al., 1991; Lee et al., 1990; Yu et al., 1999) and reviewed in (Bouchard and Schneider, 2004; Seeger, 2007)). These studies have demonstrated that HBx-transgenic mice either directly develop liver tumors or are more susceptible to treatment with carcinogens, depending on the genetic background of the mice (Kim et al., 1991; Lee et al., 1990; Yu et al., 1999). These studies also suggest that HBx can have either a direct or co-factor role in HCC development; however, considering the decades of chronic HBV infection that is usually required prior to the development of HBV-associated HCC in humans, it is more likely that HBx has a subtle co-factor role in HCC development or progression ((Lee, 1997) and reviewed in (Seeger, 2007)).

Although HBV integration into the chromosome of an HBV-infected hepatocyte is not required for HBV replication, integration of HBV DNA is often found in HBV-associated tumors ((Brechot et al., 1980; Summers et al., 1978) and reviewed in (Bonilla Guerrero and Roberts, 2005)). Because no consistent sites of HBV integration into host chromosomes have been identified, whether HBV integration into the genome of a hepatocyte affects the development of HCC is unclear (reviewed in (Chemin and Zoulim, 2009; Cougot et al., 2005)). Interestingly, truncated forms of the HBx gene are frequently found integrated into the genome of hepatocytes in HBV-infected individuals, and some studies have suggested that truncated versions of HBx are more prevalent in HBV-associated tumors as compared to adjacent non-tumor tissue (Hsia et al., 1997; Liu et al., 2008b; Tu et al., 2001; Wang et al., 2004). Many of the integrated, truncated forms of the HBx gene could potentially generate C-terminal truncated HBx mutant proteins; the C-terminus of HBx, particularly amino acids 134 through 140, is well conserved between different genotypes of HBV (Kodama et al., 1985). Truncated forms of the HBx gene that were identified in tumors and cloned into expression vectors expressed truncated HBx and activated various viral and cellular genes (Takada et al., 1990; Takada and Koike, 1990). Several other studies have also characterized C-terminal truncations of HBx. The results of one study suggested that deletion of the C-terminal region of HBx, particularly amino acids 141 to 154, increased HBx stability (Li et al., 2006); however, other studies demonstrated that deletion of the C-terminal region of HBx led to decreased steady-state HBx levels as compared to full-length HBx and that the decrease in steady-state protein levels was due to proteasomal-mediated degradation of the C-terminal mutants (Tu et al., 2001; Xu et al., 2007). Another study did not find differences in steady-state protein levels of C-terminally truncated HBx mutants as compared to full-length HBx (Liu et al., 2008a). Whether the integrated, truncations of the HBx gene that have been identified in HBV-associated liver tumors represent a selection for specific forms of HBx or are simply a consequence of the preferred integration of an aberrant, linear double-stranded form of the replicated HBV genome is unclear (Hsia et al., 1997; Liu et al., 2008b; Mason et al., 2009; Tu et al., 2001; Wang et al., 2004). Finally, although it is clear that truncations of the HBx gene can be found in many HBV-associated tumors, whether these truncations express active and stable HBx has not always been directly demonstrated in tumors in which the integrated genome was identified (Hsia et al., 1997; Liu et al., 2008b; Tu et al., 2001; Wang et al., 2004).

In the present study, we generated C-terminal HBx truncation mutants and tested the ability of these mutants to activate transcription and stimulate HBV replication in HepG2 cells. We observed that the HBx mutants with C-terminal truncations displayed different steady-state protein levels and had altered half-lives as compared to full-length HBx. We also tested these mutants for their ability to perform known HBx functions, such as stimulating HBV replication and activating the transcription factor NF- κ B, and demonstrated that C-terminal HBx truncation mutants have a diminished ability to activate NF- κ B and stimulate HBV

replication. Our observations identify regions of HBx that regulate its stability and impact on HBV replication and may also be relevant for the possible consequences of HBx-truncated HBV-genome integrants that have been observed in some HBV-associated tumors.

2. Materials and Methods

2.1 Cell culture and transfection

HepG2 cells were obtained from the American Type Culture Collection and cultured on collagen-coated tissue culture plates in Minimum Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1mM nonessential amino acids, 1mM sodium pyruvate and 10µg/ml gentamicin. The cells were maintained at 37°C in 5% CO₂. HepG2 cells were transfected using FuGENE 6 (Roche) according to the manufacturer's instructions. For all experiments, with the exception of the immunofluorescence studies, cells were co-transfected with a green fluorescent protein (GFP)-expressing plasmid to confirm equal transfection efficiency.

2.2 Antibodies

The anti-Flag-M2 antibody was purchased from Stratagene; the anti- α -tubulin antibody was purchased from Santa Cruz Biotechnology, Inc; the anti- β -actin antibody was purchased from Sigma; the anti-HBcAg antibody was purchased from DakoCytomation, Inc; and the anti-HBx antibody was purchased from Affinity BioReagents.

2.3 Plasmids

FL 1-154 HBx, containing N-terminally Flag-tagged HBx cloned into the pcDNA3.1(−) vector and 1-154 HBx, the non-Flag-tagged version, have been previously described (Clippinger and Bouchard, 2008). C-terminal truncation mutants of HBx were generated to express an N-terminal Flag-tag, FL 1-140 HBx and FL 1-131 HBx, as well as non-Flag-tagged versions, 1-140 HBx and 1-131 HBx. The forward primer used for FL 1-154 HBx, FL 1-140 HBx and FL 1-131 HBx was

5'GCCTCGAGATGGACTACAAGGACGACGATGATAAGATGGCTGCTAGGCTGTG C 3' and the forward primer used for 1-154 HBx, 1-140 HBx and 1-131 HBx was 5'

GCCTCGAGATGGCTGCTAGGCTGTGCTGC 3'. The reverse primer for FL 1-154 HBx and 1-154 HBx was 5' GCGATATGTCACTATTAGGCAGAGGTGAAAAAGTTGC 3'.

The reverse primer for FL 1-140 HBx and 1-140 HBx was 5'

GCGATATCCTATTATTTATGCCTACAGCCTCCTAG 3'. The reverse primer for FL 1-131 HBx and 1-131 HBx was 5'

GCGATATCTCACTATTAGACCTTTAATCTAATCTCCTC 3'. Construction and use of pGEMHBV (payw1.2) and pGEM*7 (payw*7), which contains an HBx-deficient HBV genome, has been previously described (Melegari et al., 1998; Scaglioni et al., 1997).

The NF- κ B luciferase reporter plasmids, NF- κ B-Luc and mtNF- κ B-Luc have been previously described (Puro and Schneider, 2007). NF- κ B-Luc encodes consensus NF- κ B sites upstream of a luciferase reporter; mtNF- κ B-Luc is identical to NF- κ B-Luc except that the NF- κ B consensus sites are mutated so that NF- κ B cannot bind and stimulate transcription. The pTAL luciferase reporter plasmid, pTAL-Luc (Clontech), contains a TATA-like promoter taken from the Herpes simplex virus thymidine kinase promoter fused upstream of the firefly luciferase gene (Luc) and is used to evaluate transcriptional activity from a minimal promoter.

2.4 Luciferase assays

HepG2 cells were transfected with increasing amounts of FL 1-154 HBx, FL 1-140 HBx, FL 1-131 HBx, or pcDNA3.1(−) (vector control) and 0.25 µg of NF- κ B-Luc or mtNF- κ B-Luc.

24 hours post-transfection, cells were washed with 1X phosphate buffered saline (PBS) and lysed in 100-200 μ L of 1X Reporter Lysis Buffer (RLB) (Promega) followed by freezing at -80°C . Cells were thawed, scraped into RLB, and pulse-centrifuged to remove debris. Protein concentrations were determined using the BioRad Protein Assay (BioRad). Lysates were assayed for luciferase activity using the Luciferase Assay System (Promega) according to the manufacturer's instructions, and luciferase activity was measured using a luminometer. Co-transfection of a GFP-expression plasmid was used to monitor equivalent transfection efficiencies.

2.5 HBV replication assay

HepG2 cells were transfected with pGEMHBV or pGEM*7 and co-transfected with pcDNA3.1(-) or one of the following: FL 1-154 HBx, 1-154 HBx, FL 1-140 HBx, 1-140 HBx, FL 1-131 HBx or 1-131 HBx. 72 hours post-transfection, HBV core particles were isolated, and HBV replication was analyzed via Southern blot as previously described (Bouchard et al., 2001b). Co-transfection of a GFP-expression plasmid was used to monitor equivalent transfection efficiencies. Blots were quantified using EZQuant software, and statistical significance between differences was determined using the Student's t-test.

2.6 Northern blot analysis

HepG2 cells were transfected with pGEMHBV or pGEM*7 and co-transfected with pcDNA3.1(-), FL 1-154 HBx, 1-154 HBx, FL 1-140 HBx, 1-140 HBx, FL 1-131 HBx or 1-131 HBx. 72 hours post-transfection, total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. The Poly(A)⁺ RNA fraction was isolated using oligo (dT)-cellulose columns (Molecular Research Center, Inc.) according to the manufacturer's instructions. HBV mRNA or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analyzed via Northern blot analysis as previously described (Bouchard et al., 2001b).

2.7 HBx half-life

HepG2 cells were transfected with 1-154 HBx, 1-140 HBx, or 1-131 HBx. 48 hours post-transfection, the cells were incubated at 37°C in 5% CO_2 for 30 minutes in Dulbecco's Modification of Eagle's Medium (Cellgro) containing 4.5 g/L glucose and 4.5 g/L sodium pyruvate without L-glutamine, L-methionine, and L-cysteine, supplemented with 10% dialyzed FBS, 10 $\mu\text{g}/\text{ml}$ gentamicin, and 20 μM MG132 (Sigma). The reversible proteasome inhibitor MG132 was added during the pulse-labeling phase to increase the amount of protein and facilitate detection of HBx mutants that have low steady-state levels of protein (Lee and Goldberg, 1998). 100 μCi of a mix of ^{35}S -labeled cysteine and methionine (Perkin Elmer) was added, and the cells were incubated at 37°C in 5% CO_2 for 90 minutes. The cells were washed three times with 1X PBS to remove unincorporated ^{35}S -labeled cysteine and methionine and MG132. Cells were then incubated in MEM, supplemented with 10% FBS, 1 mM nonessential amino acids, 1 mM sodium pyruvate and 10 $\mu\text{g}/\text{ml}$ gentamicin for the duration of the time course with cells being collected immediately after the medium change at time 0 and at 30, 60, and 120 minutes after the medium change. The cells were scraped into 1X PBS at the times indicated, pelleted, and then lysed in 100 μL of sodium dodecyl sulfate (SDS) RIPA Lysis Buffer containing 1% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 8.0, 0.5% Sodium Deoxycholate, and 1X Protease Inhibitor Cocktail (cOmplete, Roche) on ice for 10 minutes. Next, the lysates were diluted 1:10 with Lysis Buffer Diluent containing 150 mM NaCl, 50 mM Tris pH 8.0, and 1X Protease Inhibitor Cocktail. The lysates were passed through a 23-gauge syringe 15 times, and protein concentrations were determined using the BioRad Protein Assay (BioRad). Immunoprecipitations were performed with anti-Flag M2 antibody, collected using Protein G PLUS-Agarose (Santa Cruz), and washed and resolved on a 15% SDS-polyacrylamide gel

as previously described (Klein and Schneider, 1997). The dried gel was exposed to a storage phosphor screen and read using the Storm™ 840 (GE Healthcare). Results were quantified utilizing ImageQuant™ TL (GE Healthcare) software.

2.8 Western blot analysis

Cells were collected 24 or 72 hours post-transfection, washed once with 1X PBS, lysed in 2% SDS lysis buffer (2% SDS, 240 mM Tris [pH 6.8], and 10% glycerol) and equal amounts of protein were resolved on a 15% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (BioRad) and blocked for 2 hours with 5% non-fat dry milk in 1X PBS. Blots were incubated overnight at 4°C with primary antibody, washed with 1X Tris-buffered saline + 0.1% Tween 20, and then incubated with an Alexa Fluor-conjugated secondary antibody before being visualized by the quantitative Odyssey® infrared imaging system (Licor® Biosciences) according to the manufacturer's instructions. Western blot analysis was conducted using antibodies specific for β -actin, Flag, or HBV core (HBcAg). Equal loading of protein was confirmed by analysis of β -actin.

2.9 Immunofluorescence

Indirect immunofluorescence of HBx was performed as previously described (Bouchard et al., 2001a). Briefly, HepG2 cells were grown on collagen-coated glass coverslips and transfected with varying amounts of FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx. 24 hours post-transfection, coverslips were washed with 1X PBS and then washed and immersed in ice cold fixative (95% ethanol and 5% acetic acid) and maintained overnight at -20°C. Coverslips were blocked in 1X PBS + 2% bovine serum albumin (BSA) for 30 minutes at 37°C, incubated for 1 hour at 37°C with primary antibody (Anti-Flag-M2) diluted 1:100 in 1X PBS + 2% BSA, washed four times with 1X PBS + 2% BSA, and then incubated in secondary goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody diluted 1:1000 in 1X PBS + 2% BSA. Slides were mounted with VectaShield® (Vector Laboratories) and visualized using an Olympus AX70 microscope. Pictures were taken using iVision-Mac™ (BioVision Technologies) software and a Retiga EXi (QImaging) camera.

3. Results

3.1 C-terminal truncations of HBx decrease HBx steady-state levels

We generated HBx C-terminal truncation mutants and analyzed expression of these HBx truncation mutants in HepG2 cells (Figure 1A and Figure 1C). FL 1-131 HBx and FL 1-140 HBx, which have C-terminal deletions of 23 or 14 amino acids, respectively, had decreased steady-state HBx levels as compared to full-length FL 1-154 HBx (Figure 1C). Addition of the proteasome inhibitor MG132 increased the steady-state protein levels of FL 1-140 HBx and FL 1-131 HBx. These results show that the deletion of the 14 and 23 C-terminal amino acids of HBx decreased steady-state levels of HBx and that this decrease in protein levels is partially proteasome-dependent.

3.2 The C-terminus of HBx is important for HBx stability

The decrease in the steady-state level of HBx could be attributed to a variety of causes, including decreased mRNA transcription, decreased mRNA stability, decreased translation, or decreased protein stability. We first examined the effect of C-terminal HBx truncations on the level of HBx mRNA and found that the levels of HBx mRNA were not largely affected by C-terminal truncations of HBx suggesting that the C-terminus of HBx is not vital for mRNA stability (Figure 1B). We next evaluated whether the differences in steady-state protein levels were a result of decreased protein stability. HepG2 cells were transfected with

pcDNA3.1(-), 1-154 HBx, 1-140 HBx, or 1-131 HBx and treated as described in Materials and Methods. In these experiments, we incubated cells in media containing the reversible proteasome inhibitor MG132 before and during ³⁵S-labeled cysteine and methionine exposure; MG132 was added to facilitate detection of the mutant HBx proteins, which were otherwise expressed at low steady-state levels. ³⁵S-labeled cysteine and methionine and MG132 were removed at the same time, and protein degradation was allowed to proceed normally. Cells were collected 0, 30, 60, and 120 minutes after ³⁵S-labeled cysteine and methionine and MG132 were removed. Although protein degradation and HBx stability was only analyzed for two hours, this analysis demonstrated that the half-life of wild type 1-154 HBx was approximately 120 minutes, possibly longer. A summary of the half-life analyses, quantified as outlined in Material and Methods, is shown (Figure 1D). Importantly, we observed shorter half-lives for 1-131 HBx and 1-140 HBx. The half-life for 1-131 HBx was 80 minutes, and the half-life of 1-140 HBx was 100 minutes (Figure 1D). While the half life of HBx and the HBx mutants may have been slightly affected by the necessity of using MG132 to help stabilize steady-state levels and subsequent detection of mutant HBx proteins, these results none-the-less demonstrate that the C-terminus of HBx is critical for HBx stability in HepG2 cells.

3.3 C-terminal truncations of HBx impair HBx activation of NF-κB and a minimal promoter

We next analyzed whether the C-terminus of HBx is required for HBx activation of cellular transcription pathways. HBx activates a variety of transcription factors, including NF-κB (Chirillo et al., 1996; Kwee et al., 1992; Lucito and Schneider, 1992; Mahe et al., 1991; Su and Schneider, 1996; Waris et al., 2001). Using an NF-κB-dependent luciferase reporter assay system, we examined the effect of C-terminal truncations on HBx activation of NF-κB. HepG2 cells were transfected with increasing amounts of FL 1-154 HBx, FL 1-140 HBx, FL 1-131 HBx, or pcDNA3.1(-) and 0.25 μg NF-κB-Luc or mtNF-κB-Luc. Cells were collected 24 hours post-transfection. FL 1-154 HBx activated NF-κB between 2- and 10-fold higher than pcDNA3.1(-) in a dose-dependent manner (Figure 2A). FL 1-140 HBx also activated NF-κB, though to a lesser extent, ranging from 1- to 3.5-fold (Figure 2A). FL 1-131 HBx did not activate NF-κB (Figure 2A). Additionally, we accounted for differences in the stability of FL 1-140 HBx and FL 1-131 HBx by increasing the amount of HBx plasmid expressing the truncated HBx proteins transfected into cells and equalized the steady-state levels of HBx proteins (Figure 2D); the HBx truncation mutants were still unable to activate NF-κB to the same level as wild-type HBx (Figure 2A). For example, 0.125 μg of FL 1-154 HBx induced NF-κB activity 8-fold, whereas 0.5 μg FL 1-140 HBx increased NF-κB activity 3.5-fold (Figure 2A). 0.063 μg FL 1-154 HBx induced NF-κB activity 2-fold, whereas none of the transfected amounts of FL 1-131 HBx were able to induce NF-κB activity above pcDNA3.1(-) levels (Figure 2A). These results suggest that while FL 1-140 HBx can still activate NF-κB, it does not increase NF-κB activity to a degree similar to that of wild-type HBx, demonstrating that the 14 C-terminal amino acids of HBx are important for HBx activation of NF-κB in HepG2 cells. We consistently observed that 0.50 μg of FL 1-154X activated NF-κB to levels that were lower than 0.125 μg and 0.25 μg of FL1-154X; the reason for this decrease in NF-κB activity at higher HBx levels is unknown and the subject of ongoing studies. We also tested mtNF-κB-Luc, a luciferase reporter gene in which the NF-κB binding sites are mutated such that NF-κB cannot bind to activate the promoter; this construct was used as a negative control for our NF-κB-Luc reporter. We found that none of our HBx constructs could activate mtNF-κB-Luc (Figure 2B). Finally, we examined the ability of these HBx truncation mutants to activate TAL, a minimal, TATA-like promoter region derived from the HSV-TK promoter. FL 1-154 HBx activated Tal approximately 2-fold while little to no activation of Tal was detected in HepG2 cells that were transfected with FL 1-140 HBx. FL 1-131 HBx slightly but consistently inhibited the activation of Tal (Figure 2C). Overall, our results demonstrate that C-terminal

deletions of HBx decreased its ability to activate transcriptional NF- κ B- and Tal-dependent promoters.

3.4 Deletion of the C-terminus of HBx does not affect cellular localization

We next examined the effect of C-terminal deletions of HBx on HBx localization in HepG2 cells; localization of HBx might impact its functions. HepG2 cells were transfected with 0.5 μ g of FL 1-154 HBx, 1.0 μ g or 2.0 μ g of FL 1-140 HBx, or 1.0 μ g or 2.0 μ g of FL 1-131 HBx and collected 24 hours post-transfection. The cells were labeled with anti-Flag antibody, and a FITC-conjugated secondary antibody. FL 1-154 HBx, FL 1-140 HBx, and FL 1-131 HBx displayed a similar, predominately cytosolic, localization of HBx (Figure 3). These results suggest that truncations of the 14 and 23 C-terminal amino acids of HBx do not affect the localization of HBx in HepG2 cells.

3.5 C-terminal deletions of HBx led to decreased HBV replication in HepG2 cells

We next determined whether the C-terminus of HBx is required for HBx-induced stimulation of HBV replication in HepG2 cells. Previous studies demonstrated that HBx is important for HBV replication in HepG2 cells (Melegari et al., 1998). Therefore, we examined the effects of C-terminal truncations of HBx on HBV replication. HepG2 cells were transfected with pGEMHBV or an HBx-deficient genome, pGEM*7, and co-transfected with pcDNA3.1(-), FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx, and the levels of HBV replication were evaluated. FL 1-154 HBx restored HBV replication derived from pGEM*7 to levels that were similar to HBV replication in pGEMHBV-transfected cells despite using relatively low amounts of the FL 1-154 HBx-expression plasmid (Figure 4A and Figure 4C, compare lanes 2 and 4). However, FL 1-140 HBx and FL 1-131 HBx could not rescue HBV replication in pGEM*7-transfected HepG2 cells even when FL1-131 HBx or FL1-140 were expressed at levels equivalent or greater than wildtype HBx in parallel assays of wildtype HBx rescue of HBV replication in pGEM*7-transfected HepG2 cells. While differences between pGEMHBV-transfected cells and pGEM*7 + FL 1-154 HBx-transfected cells were not found to be statistically significant (Figure 4A, lanes 1, 3, and 4), differences between pGEMHBV-transfected cells and pGEM*7 + FL 1-140 HBx or pGEM*7 + FL 1-131 HBx-transfected cells were statistically different (Figure 4A, lanes 1, 5, and 6). We also examined the effect of C-terminal HBx truncations on the levels of pregenomic RNA (pgRNA) and HBV surface antigens (HBsAg) RNA and found that the levels were not largely affected by C-terminal truncations of HBx suggesting that the C-terminus of HBx is required for HBV DNA replication but not for the transcription of viral RNAs (Figure 4B). While the C-terminal HBx truncations could not rescue HBV replication, the absence of HBx also did not affect the levels of HBV core protein (HBcAg) expression (Figure 4D). Overall, these results demonstrate that the C-terminus of HBx is required for HBx-stimulation of HBV replication but not for synthesis of HBV RNAs or other HBV proteins in HepG2 cells. The inability to detect HBx in pGEMHBV-transfected cells (Figure 4C, lane 1) is consistent with the low levels of HBx that is expressed in the context of HBV replication and provides further support for the notion that the inability of these C-terminally truncated HBx to rescue HBV replication is unlikely related to low expression levels but rather that the C-terminus of HBx has an important role in HBV replication.

4. Discussion

Worldwide, chronic HBV infections are the leading cause of HCC (reviewed in (Block et al., 2003; Seeger, 2007)). Although it is clear that HBV-associated inflammation and destruction of HBV-infected hepatocytes by the immune response of the host is an important contributor to the development of HCC, HBx is also likely to impact liver cancer

development as well as HBV replication in individuals who are infected with HBV (reviewed in (Bouchard and Schneider, 2004; Seeger, 2007)). HBx can modulate various cellular processes including cell transcription, proliferation, and apoptosis pathways (reviewed in (Bouchard and Schneider, 2004)). In the present study, we have begun to identify regions of HBx that are important for its activities and have characterized the impact of C-terminal truncations of HBx on HBx stability and HBx-mediated transcription activation and HBV replication. We demonstrated that C-terminal truncations of 14 and 23 amino acids decreased HBx steady-state levels (Figure 1); this decreased steady state level was at least partially dependent on proteasome activity. Deletion of amino acids 141 to 154 decreased HBx stability from 2 hours (wild-type 1-154 HBx) to 100 minutes (mutant 1-140 HBx) and deletion of amino acids 132 to 154 decreased HBx stability to 80 minutes (mutant 1-131 HBx) (Figure 1D). The decreased stability of the truncated HBx mutants meant that we had to overcome decreased steady-state HBx levels to test the functionality of the C-terminal HBx truncation mutants. This was accomplished by increasing the amounts of transfected HBx-truncation containing plasmids relative to the HBx wildtype expression plasmid. Nearly undetectable levels of FL 1-154 HBx led to increased NF- κ B activity and stimulated HBV replication (Figures 2A and 4A). In contrast, when FL 1-140 HBx and FL 1-131 HBx were expressed at levels that were similar to or greater than the level of FL 1-154 HBx, neither FL 1-140 HBx nor FL 1-131 HBx could stimulate NF- κ B activity or HBV replication to levels that were equivalent to FL 1-154 HBx. Although FL 1-140 HBx retained some ability to activate NF- κ B, increased expression of FL 1-140 HBx did not enhance this effect, suggesting that amino acids 141 to 154 of HBx are required for complete HBx-induced activation of NF- κ B. Finally, although C-terminal HBx truncation affected HBx activities, amino acids 131 to 154 do not control the cellular localization of HBx (Figure 3).

The results of some studies have suggested that disruption of the HBx C-terminus does not affect the ability of HBx to transactivate a simian vacuolating virus 40 (SV40) promoter (Takada et al., 1990; Takada and Koike, 1990). However, there have been other reports that suggest the C-terminus is important for HBx-induced transcriptional transactivation of HBV enhancer I, the SV40 promoter, activating protein 1 (AP-1), and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP-10) (Li et al., 2006; Murakami et al., 1994; Tang et al., 2005). HBx amino acids 88 to 154 are important for HBx-induced transcriptional transactivation of AP-1 and HBV enhancer 1 and for HBV replication in HepG2 cells (Tang et al., 2005). Previously published results that have identified the C-terminal region of HBx as being important for the ability of HBx to activation transcription pathways are consistent with our observations; we have demonstrated that amino acids 141 to 154 are important for HBx-induced transactivation of NF- κ B and HBx-induced stimulation of HBV replication in HepG2 cells (Figures 2 and 4). Our study is the first to find that, despite over-expressing the C-terminal HBx mutants that lack amino acids 131 to 154 and 140 to 154 so as to equalize steady-state protein levels to those of wildtype HBx, there was still no activation of NF- κ B or a minimal transcription promoter or stimulation of HBV replication in HepG2 cells (Figure 2 and Figure 4A). These observations confirm that the C-terminus of HBx is required for HBV replication.

Some recent studies have focused on generating panels of HBx mutants and evaluating the effects of these mutations on HBx function. Tu and colleagues created a series of HBx mutants that were PCR-amplified HBx DNA sequences derived from patient sera and tumor tissue samples and cloned into an expression vector. In these studies, C-terminal deletions led to decreased steady-state HBx levels (Tu et al., 2001). Their studies in murine hepatocyte (α -ML) cells indicated that HBx mutants with C-terminal truncations greater than 18 amino acids could not transactivate a cyclic AMP response element (CRE) luciferase reporter construct (Tu et al., 2001). Our results are in agreement with these studies

and further narrow the region of HBx important for its transcriptional transactivation activity, at least for a basal transcription promoter and activation of NF- κ B. We show that amino acids 141 to 154 are important for the transcriptional transactivation activity of HBx and that the C-terminus of HBx is important for protein stability. The results of one study have demonstrated that deletion of amino acids 141 to 154 increased HBx stability (Li et al., 2006). The reason for differences between the studies of Li *et al.* as compared to our studies and those of Tu *et al.* are not clear but could reflect cell-line specific effects on HBx stability. In the study by Li *et al.*, tetracycline-inducible HBx expressing cells were generated in with the alpha-mouse liver 12 (AML12) cell line, whereas our studies were conducted in HepG2 cells. It will be important in future studies to determine the impact of HBx truncations on the steady-state levels of HBx in normal human hepatocytes.

The results of several studies have demonstrated that HBV viral genomes carrying C-terminal deletions of HBx are frequently found integrated in the genome of HBV-associated liver tumor cells (Brechot et al., 1980; Hsia et al., 1997; Liu et al., 2008b; Tu et al., 2001; Wang et al., 2004). These observations have led some to suggest that C-terminal truncations of HBx may promote tumor formation and the development of HBV-associated HCC development (Liu et al., 2008b; Ma et al., 2008; Sirma et al., 1999). Although we have not evaluated the potential transformation activity of our C-terminally truncated HBx mutants, it is possible that disruption of the C-terminus leads to differential HBx activities, which could promote tumor formation. Unfortunately, several of the studies that demonstrated an association between integrated HBx C-terminal mutants and tumor tissues did not directly evaluate the steady-state protein levels of the various HBx mutants directly in the tumors, and it remains unknown whether these HBx proteins were expressed or active in the tumors or contributed to transformation processes (Hsia et al., 1997; Liu et al., 2008b; Tu et al., 2001; Wang et al., 2004). Our demonstrations that C-terminal truncations of HBx decreased protein stability and impacted HBx transcriptional activities and stimulation of HBV replication suggest caution when interpreting the results of HBx gene integration studies in the absence of directly demonstrating expression of functional, active HBx in tumors that contain integrated, C-terminal truncations of HBx.

Whether C-terminal truncation of HBx impact HBx regulation of other cellular signal transduction pathways in HepG2 cells and normal hepatocytes is the subject of our ongoing studies. For example, HBx localizes to mitochondria, and mitochondrial functions are important for HBx modulation of cellular apoptosis, proliferation, and calcium signaling pathways as well as HBx stimulation of HBV replication; the affect of HBx C-terminal truncations on these HBx activities is unknown (Bouchard et al., 2001a; Clippinger and Bouchard, 2008; Clippinger et al., 2009; Gearhart and Bouchard, 2010a; Gearhart and Bouchard, 2010b; McClain et al., 2007). Identifying regions of HBx that control its stability and functions may help elucidate mechanisms that are associated with the role of HBx during HBV replication and in the development of HBV-associated HCC.

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FIGURE 1.

Deletion of the HBx C-terminus leads to decreased protein expression and stability. (A) Schematic of full-length HBx and truncation mutants created for this study. (B) HepG2 cells were transfected with pcDNA3.1(-) control vector or one of the following: FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx. 72 hours post-transfection, mRNA was extracted and visualized using Northern blot analysis. GAPDH was used as a loading control. Untransfected or pcDNA3.1 transfected HepG2 cells expressed no RNAs that cross-reacted with the HBx-specific probe (not shown). (C) HepG2 cells were transfected with 2.0 μ g of FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx. 48 hours post-transfection, cells were treated with 20 μ M MG132 or DMSO (vehicle control) for 6 hours. Samples were collected and resolved via Western blot analysis for Flag(HBx) and β -actin. (D) HepG2 cells were transfected with FL 1-154 HBx (154X), FL 1-140 HBx (140X), or FL 1-131 HBx (131X) and pulse-chase experiments were carried out as described in the Materials and Methods. The table shows the quantification of those results where expression of 154X, 140X, or 131X at time 0 was set as 100%; expression levels of 154X, 140X, or 131X at subsequent time points are expressed as a percentage of the level of each relative to their expression at time zero.

FIGURE 2.

Deletion of the HBx C-terminus leads to decreased NF-κB activation. (A-C) HepG2 cells were transfected with FL 1-154 HBx (154X), FL 1-140 HBx (140X), or FL 1-131 HBx (131X) and one of the following luciferase reporter gene constructs: NF-κB-Luc, mtNF-κB-Luc, or pTal-Luc and luciferase assays were conducted as described in Materials and Methods. The graphs represent luciferase activity equilibrated by protein concentration and expressed as a fold difference as compared to samples transfected with pcDNA 3.1 (-). The error bars represent standard error. The graphs represent the results of three experiments each performed in triplicate. (D) HepG2 cells were transfected with pcDNA3.1(-), FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx. Cells were collected 24 hours post-transfection and resolved via Western blot analysis for Flag (HBx) and β-actin. pc, pcDNA3.1(-); 154X, FL 1-154 HBx; 140X, FL 1-140 HBx; 131X, FL 1-131 HBx.

FIGURE 3.

C-terminal deletions of HBx does not impact the cellular localization of HBx. (A-E) HepG2 cells were transfected with 1 µg of FL 1-131 HBx or FL 1-140 HBx (A and C), 2 µg of FL 1-131 HBx or FL 1-140 HBx (B and D), or 0.5 µg of FL 1-154 HBx (E). Indirect immunofluorescence was performed by labeling transfected cells with an anti-Flag antibody and a secondary FITC-conjugated antibody.

FIGURE 4.

C-terminal truncation of HBx leads to decreased HBV replication. (A-D) HepG2 cells were transfected with pGEMHBV or pGEM*7 and co-transfected with pcDNA3.1(-) control vector or one of the following: FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx. The numbers above the blots indicate the μ g amount of pcDNA3.1(-), FL 1-154 HBx, FL 1-140 HBx, or FL 1-131 HBx plasmid transfected. (A) 72 hours post-transfection, HBV core particles were isolated and HBV replication was analyzed via Southern blot. *, statistically significant fold difference between HBV and sample marked as determined using Student's *t* test ($P \leq 0.01$). RC, relaxed circular; DL, double-stranded linear; SL, single-stranded linear. (B) 72 hours post-transfection, mRNA was extracted and visualized using Northern blot analysis. GAPDH was used as a loading control. pgRNA, pre-genomic RNA; HBcAg, HBV core antigen, HBsAg; HBV surface antigens. (C-D) 72 hours post-transfection, cells were collected and resolved via Western blot analysis for B-actin, Flag, and HBcAg. HBV, pGEMHBV; HBV*7, pGEM*7; pc, pcDNA3.1(-); 154X, FL 1-154 HBx; 140X, FL 1-140 HBx; 131X, FL 1-131 HBx.