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Hepatitis B Virus X Protein Stimulates Viral Genome Replication via a DDB1-Dependent Pathway Distinct from That Leading to Cell Death

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The hepatitis B virus (HBV) X protein (HBx) is essential for virus infection and has been implicated in the development of liver cancer associated with chronic infection. HBx can interact with a number of cellular proteins, and in cell culture, it exhibits pleiotropic activities, among which is its ability to interfere with cell viability and stimulate HBV replication. Previous work has demonstrated that HBx affects cell viability by a mechanism that requires its binding to DDB1, a highly conserved protein implicated in DNA repair and cell cycle regulation. We now show that an interaction with DDB1 is also needed for HBx to stimulate HBV genome replication. Thus, HBx point mutants defective for DDB1 binding fail to complement the low level of replication of an HBx-deficient HBV genome when provided in *trans*, and one such mutant regains activity when directly fused to DDB1. Furthermore, DDB1 depletion by RNA interference specifically compromises replication of wild-type HBV, indicating that HBx produced from the viral genome also functions in a DDB1-dependent fashion. We also show that HBx in association with DDB1 acts in the nucleus and stimulates HBV replication mainly by enhancing viral mRNA levels, regardless of whether the protein is expressed from the HBV genome itself or supplied in *trans*. Interestingly, whereas HBx induces cell death in both HepG2 and Huh-7 hepatoma cell lines, it enhances HBV replication only in HepG2 cells, suggesting that the two activities involve distinct DDB1-dependent pathways.

Chronic infection by hepatitis B virus (HBV) affects 350 million people worldwide and is a major causative agent of liver diseases, including cirrhosis and hepatocellular carcinoma, one of the most common cancers in humans (27). HBV belongs to the *Hepadnaviridae* family of small enveloped DNA viruses that replicate primarily in the livers of their hosts and exhibit similarities to retroviruses. This family also contains rodent viruses, such as woodchuck hepatitis virus and ground squirrel hepatitis virus, as well as more distantly related members infecting avian species. While all mammalian hepadnaviruses cause liver cancer in their hosts, avian viruses do not (13).

The main features of the hepadnavirus replication cycle are quite well understood (for a review, see reference 14). Upon infection, the 3.2-kb circular, partially double-stranded viral genome is transported into the cell nucleus, where it is converted into a covalently closed circular DNA. The covalently closed circular DNA serves as a template for transcription by host cell RNA polymerase II of four major viral RNA species, including the more than full-length pregenomic RNA. The pregenomic RNA is then reverse transcribed into DNA replicative intermediates in the cytoplasm within immature viral core particles by the virally encoded polymerase.

Mammalian hepadnaviruses encode a small regulatory protein, known as the X protein, for which no obvious counterpart exists in the nononcogenic avian hepatitis viruses. The X protein is well conserved among all mammalian hepadnaviruses, is expressed during infection both in humans (32) and in woodchucks (9, 16), and is required for the viral life cycle in the woodchuck (6, 29, 42, 44). Several lines of evidence suggest that the X protein may also contribute to hepatocellular carcinoma development (reviewed in reference 1). However, the basis for X protein function in either process remains elusive.

The HBV and woodchuck hepatitis virus X proteins (also referred to as HBx and WHx, respectively) localize to both the cytoplasm and the nucleus (8, 12, 30, 34), and in tissue culture cells they exhibit similar pleiotropic activities, affecting transcription, DNA repair, cell growth, and apoptotic cell death (for reviews, see references 1 and 26). HBx and WHx can also stimulate viral replication in some human hepatoma cell lines, and they have been reported to do so either by activating viral transcription (7, 25, 42) or by enhancing the reverse transcription activity of the viral polymerase (4, 17). The proteins are thought to mediate these various activities through interactions with cellular factors. Indeed, an impressive number of potential cytoplasmic and nuclear targets have been reported to bind HBx or WHx (26). Among these, DDB1 is the only candidate for which interaction with human, woodchuck, and ground squirrel X proteins has been documented (31), and there is evidence that this interaction is critical for efficient viral infection in woodchucks (29).

DDB1 was originally identified as a 127-kDa protein that associates with DDB2 to form the UV-DDB complex that functions in nucleotide excision repair (reviewed in reference 38). However, DDB1 has more recently also been implicated in other pathways. DDB1 was found to be integrated into multisubunit complexes containing cullin-4A and other proteins and displaying E3 ligase activity (15), suggesting that it may be involved in selecting spe-

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cific targets for ubiquitin-dependent proteolysis (37). Evidence has also been presented that DDB1 is part of the STAGA histone acetylase complex (24). DDB1 is highly conserved during evolution, and genetic studies in *Schizosaccharomyces pombe*, which lacks any obvious DDB2 homologue, suggest that it plays a role in S phase progression (2, 22).

Previous work has established that HBx induces cell death by binding to DDB1 and that this activity involves a DDB2-independent nuclear function of DDB1 (3, 20). A DDB1-binding-dependent cytotoxic activity has also been reported for the WHx protein (30), suggesting that this feature is conserved. In the present study, we investigated the importance of the HBx-DDB1 interaction for HBV genome replication in cultured hepatoma cell lines.

MATERIALS AND METHODS

Expression constructs. All recombinant DNA work was carried out following standard procedures. Details of the plasmid constructions are available upon request.

The replication-competent wild-type HBV construct (payw1.2) and the HBx-deficient derivative (payw*7) carrying a translational termination signal after codon 7 in the X gene were a kind gift of Robert J. Schneider and colleagues (New York University School of Medicine) and have been described elsewhere (25). All mammalian expression plasmids encoding HBx and variants thereof have been described previously. HBx and the point mutant derivatives were expressed from the EBO-76PL episomal vector (21) under the control of the simian virus 40 early promoter (Fig. 1B) and from pSRαS (3) under the control of the SRα promoter (Fig. 1A; also see Fig. 3B). HBx and the HBx(R96E) point mutant expressed as a native protein or as an amino-terminal fusion to wild-type DDB1 (R96E-DDB1 in Fig. 1C) or to the DDB1(i947) mutant [R96E-DDB1(i947) in Fig. 1C] were produced from EBS-PL (20) under the control of the SRα promoter. GFP-HBx, GFP-HBx(R96E), and the GFP-HBx variants bearing a heterologous nuclear localization signal (NLSGFP-HBx) or nuclear export signal (NESGFP-HBx) at the amino terminus were expressed from pSRαS (see Fig. 3A and 4B) (3). GFP-HBx and GFP-HBx(R96E) were expressed from lentivirus vectors (see Fig. 4C). The GFP-HBx and GFP-HBx(R96E) coding sequences were excised from pSRαS and introduced into the optimized human immunodeficiency virus-based vector pWPT (45) downstream of the EF1α short promoter (kindly provided by Tuan-Huy Nguyen and Didier Trono, Geneva, Switzerland). To allow lentivirus-mediated delivery of the DDB1-specific small interfering RNA (siRNA), the H1-DDB1-siRNA cassette containing regulatory sequences from pSUPER and originally constructed in EBOB-PL (20) was transferred into pLV-TH carrying a green fluorescent protein (GFP) marker (39). The LV-THsi/p53 construct directing the synthesis of siRNA against p53 was a kind gift of Maciej Wiznerowicz and Didier Trono (Geneva, Switzerland) and has been described elsewhere (39). All recombinant lentiviruses were produced by transient transfection of 293T exactly as described previously (45). When needed, GFP produced either from pEGFP-C1 (Clontech) or from the GFP open reading frame of pEGFP-N1 (Clontech) cloned into pSRαS was used to assess transfection efficiencies.

Cell culture, transfection, and transduction with lentivirus vectors. The human hepatoma HepG2 cell line obtained from the American Type Culture Collection and the Huh-7 cell line were grown at 37°C in the presence of 5% CO_2 in modified Eagle's medium (MEM) (Invitrogen) supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 10% (vol/vol) fetal calf serum (Chemie Brunschwig). HeLa and 293T cells were grown under identical conditions except that Dulbecco's MEM was used in place of MEM.

HepG2 or Huh-7 cells that had been plated the previous day at a density of $\sim 10^6$ total cells per 30-mm-diameter well in normal or collagen-coated six-well culture dishes were transfected using the FuGENE 6 reagent (Roche) according to the manufacturer's instructions. An expression plasmid for GFP or red fluorescent protein was cotransfected (10% of total DNA) to assess transfection efficiencies; at 24 h posttransfection, cells were trypsinized, and a fraction (usually 1/10) was scanned by fluorescence-activated cell sorter (FACS) for GFP fluorescence. Transfection efficiencies were ~5% for HepG2 cells and ~20% for Huh-7 cells, with variations of <20% within any single experiment. For transduction, cells were plated, and after 16 h, medium containing recombinant lentivirus vectors was added. Following 24 h of incubation, the cells were washed with phosphate-buffered saline and split. Two days later, the cells were trypsinized and a fraction (usually 1/10) was scanned by FACS for GFP fluorescence to assess transduction efficiencies; transduction efficiencies ranging from 95 to close to 100% were obtained. The remaining cells were transfected with HBV genomic DNA as described above (Fig. 2).

Analysis of HBV DNA replication and transcription. Viral replication was assessed by purification of cytoplasmic core particles exactly as previously described (25). Briefly, HepG2 and Huh-7 cells transfected with HBV DNA alone or together with HBx expression plasmids as described above were replated 24 h posttransfection at high density (>80% confluency) and cultured for 2 days. We noticed that when cells were plated at lower density, HBV replication showed reduction or loss of HBx dependency in HepG2 cells (data not shown). Three days after transfection, the cells were harvested, and cytoplasmic core particle-associated HBV DNA was prepared and analyzed by Southern blot hybridization as described previously (25). Radioactively ^{32}P-labeled probes prepared from full-length HBV genomic DNA were generated by using the Redi-prime labeling kit (Amersham) as described by the manufacturer. The viral transcripts were analyzed (Fig. 3B) by Northern blot hybridization of total cellular RNA extracted using Trizol reagent (Invitrogen). Equal amounts of RNA from each sample were separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N+ nylon membrane (Amersham). Hybridization was performed using the same probe described above. The hybridization signals corresponding to the single-stranded DNA (Fig. 3B, upper blot) and pregenomic RNA (lower blot) species were quantitated using a Bio-Rad phosphorimager and Quantity One software. The subtracted background intensity for each band was determined from a rectangle of equal size drawn immediately above the rectangle for each DNA or RNA band. The pregenomic RNA signals were normalized to β-actin.

Western blot analysis. The Western blot analyses presented in Fig. 1C, 2B, and 4B were performed with whole-cell extracts prepared as described previously (21). The membranes were probed with 1:500 anti-DDB1 monoclonal antibody (Zymed Laboratories), 1:2,000 anti-human p53 antibody DO1 (sc-126; Santa Cruz Biotechnology), 1:100 anti-HBx monoclonal antibody (monoclonal antibody 8419; Chemicon), or an α-tubulin-specific antibody (Sigma-Aldrich).

RESULTS

Binding to DDB1 is required for HBx stimulation of HBV replication. To assess the role of the HBx-DDB1 interaction in HBV replication, we made use of an HBx-dependent replication system in tissue culture cells in which the HBx function can be provided in *trans* (4, 7, 25). Human hepatoma HepG2 cells were transfected with equivalent amounts of either a wild-type HBV genome or an HBx-deficient derivative [HBV(ΔX)] carrying a stop codon at position 8 of the HBx open reading frame (25) (kindly provided by R. J. Schneider and colleagues), with or without cotransfection of an HBx expression plasmid. Three days later, cytoplasmic viral core particles were purified, and the amount of associated viral DNA replicative intermediates was assessed by Southern blot hybridization. In these experiments, the HBx-deficient HBV genome shows up to 20-fold-reduced levels of replication compared to the wild type (Fig. 1A, compare lane 1 to lane 2 and lane 5 to lane 8; also see below), which is in accordance with previous studies (4, 25). Cotransfection of an HBx expression plasmid restores replication of the mutant HBV genome to the normal level while having no effect on replication of the wild-type genome (Fig. 1A, compare lane 4 to lane 1 and lane 6 to lane 5). Hence, the lower and probably more physiological amount of HBx produced from the viral template suffices for HBx to fully exert its stimulatory effect. In contrast, cotransfection of the HBx(R96E) point mutant, which lacks DDB1-binding and concomitant cytotoxic activities (21), affects neither mutant nor wild-type HBV replication (Fig. 1A, lanes 3 and 7). This suggests that HBx(R96E) does not interfere with the activity of the virally encoded proteins and that an interaction with DDB1 is required for HBx to promote HBV DNA replication.

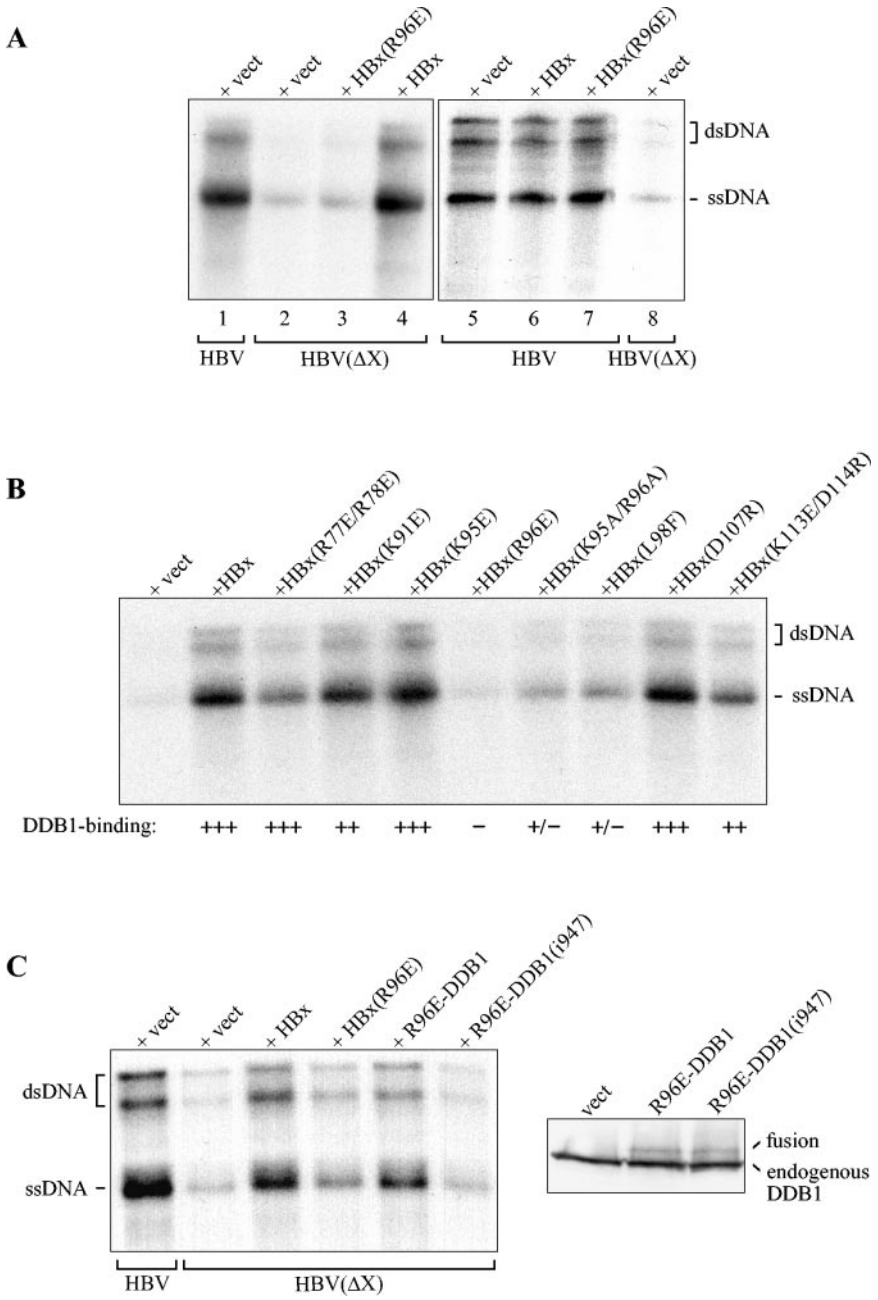

FIG. 1. HBx expressed in *trans* stimulates HBV genome replication through interaction with DDB1. HepG2 cells were transfected with a greater-than-unit-length HBV genomic construct (HBV) or with a derivative bearing a stop codon in the X gene to abolish expression of HBx [HBV(ΔX)], and equal amounts of the indicated HBx expression plasmids or the corresponding empty vector (vect). A GFP gene was cotransfected to assess comparable transfection efficiencies by FACS analysis (data not shown). Three days after transfection, cytoplasmic HBV core particles were isolated from equal numbers of cells, and the amount of associated HBV DNA replicative intermediates was assessed by Southern blot analysis. Hybridization was performed with ^{32}P-labeled probes prepared from full-length HBV genomic DNA. (A) HBx and the DDB1-binding-defective HBx(R96E) point mutant were individually tested for their effects on replication of the wild-type (left) or the HBX-deficient (right) HBV genome when expressed in *trans* from a recombinant vector. The single-stranded (ssDNA) and double-stranded (dsDNA) HBV DNA replicative forms are indicated on the right. The results are representative of three independent transfection experiments. (B) HBx mutants with single or double amino acid substitutions were tested for complementation of the mutation in HBV(ΔX). The DDB1-binding abilities of the wild-type and mutant proteins as measured in a yeast two-hybrid assay were determined previously (21) and are summarized below the panel. All mutants were shown before to accumulate to protein levels similar to those of the wild type (21). Symbols: +++, wild-type interaction; +/−, very weak interaction; −, no detectable interaction. (C) The nonfunctional HBx(R96E) mutant, which cannot interact with the endogenous DDB1 protein, was tested for activity in the complementation assay when expressed as a fusion to wild-type DDB1 (R96E-DDB1) or to the DDB1(i947) insertion mutant that is selectively defective for HBx binding [R96E-DDB1(i947)] (20). Previous work has shown that a covalent link between HBx(R96E) and DDB1 restores cytotoxic activity to the HBx mutant by acting as a clamp forcing the two protein moieties into their natural interaction and that the i947 mutation in DDB1 prevents this from occurring (20). Results from one of three independent transfection experiments are shown. On the right is a Western blot analysis of the fusion proteins. Equal amounts of whole-cell extracts prepared from HepG2 cells transfected with the indicated plasmids were resolved by gel electrophoresis, and immunoblot analysis was performed with antibodies to DDB1. The signal corresponding to endogenous DDB1 serves as a control for loading. The relatively weak signals obtained for the fusion proteins compared to endogenous DDB1 are due to low (~10%) transfection efficiencies of HepG2 cells.

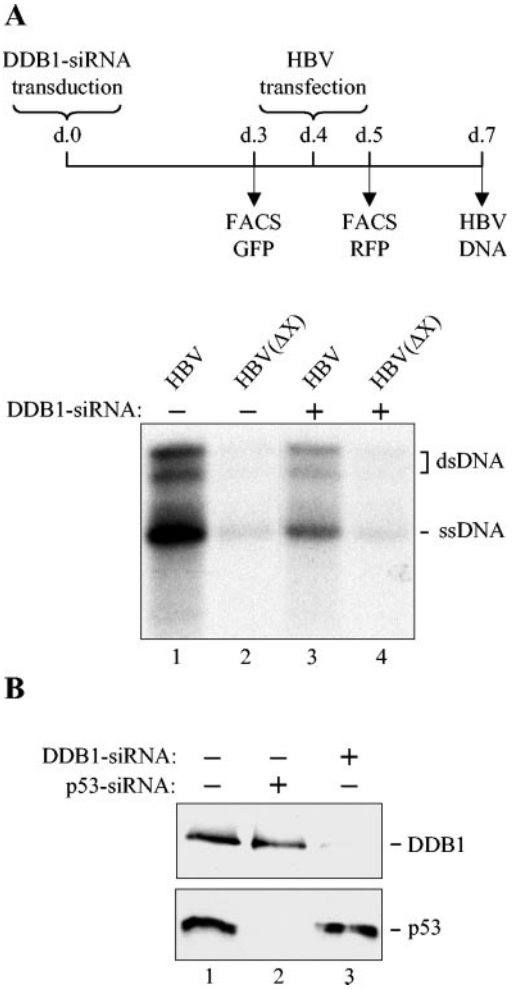

FIG. 2. HBx produced from the HBV genome shows decreased activity upon RNAi-mediated downregulation of endogenous DDB1 gene expression. (A) HepG2 cells were transduced with a control lentivirus vector expressing GFP alone (–) or with a derivative (+) that directs the synthesis of a DDB1-specific small interfering RNA (DDB1-siRNA). FACS analysis performed on day 3 (d.3) showed similar transduction efficiencies ranging from 95 to close to 100% (data not shown). On day 4, the cells were transfected with wild-type HBV or the HBV(ΔX) genomic construct. A red fluorescent protein (RFP) gene was cotransfected to assess comparable transfection efficiencies by FACS analysis 1 day later (data not shown). Viral DNA replication was assessed by Southern blot analysis 3 days posttransfection on day 7 as described in the legend to Fig. 1. Results from one of two independent experiments are shown. The single-stranded (ssDNA) and double-stranded (dsDNA) HBV DNA replicative forms are indicated on the right. (B) Western blot analysis demonstrating selective depletion of DDB1 by siRNA. HepG2 cells were transduced with a control lentivirus vector containing GFP alone (lane 1) or with a derivative expressing an siRNA against p53 (p53-siRNA) (lane 2) or DDB1 (DDB1-SiRNA) (lane 3). Cell lysates were prepared 6 days after transduction, and equal amounts of protein per sample were resolved by gel electrophoresis. Immunoblot analysis was performed with antibodies to DDB1 (top) or p53 (bottom). Downregulation of endogenous p53 expression had no effect on the replication of the wild-type or HBx mutant HBV genome (data not shown).

To further examine the correlation between DDB1 binding and viral replication, we analyzed a series of additional HBx substitution mutants that were tested previously for DDB1-binding and cytotoxic activities (21). Figure 1B shows that all the mutants complement the absence of HBx in HBV replication with efficiencies that parallel their ability to bind DDB1 and induce cell death (21), with the possible exception of HBx(R77E/R78E). This mutant, which behaves like the wild type with respect to DDB1 binding and cytotoxicity (21), shows reduced activity in the replication assay and may therefore be compromised in a function specifically required for HBV replication other than DDB1 binding.

In previous work, we showed that mutant HBx(R96E), which cannot interact with endogenous DDB1, regains cytotoxic properties when directly fused to DDB1 and that this is due to the covalent link acting as a "clamp" forcing the two protein moieties into their natural interaction (20). This does not occur when HBx(R96E) is linked to DDB1(i947), a four-amino-acid insertion mutant specifically impaired for HBx binding, presumably because in this case the interaction between the two mutant proteins is too severely compromised (20). We examined the abilities of the fusion constructs to substitute for wild-type HBx in viral replication. Figure 1C shows that, when expressed at comparable levels, the HBx(R96E)-DDB1 fusion protein stimulates replication of the HBx-deficient HBV genome to levels close to those observed upon cotransfection of wild-type HBx, whereas the HBx(R96E)-DDB1(i947) fusion is inactive in this assay. These results strengthen the notion that binding to DDB1 is required for HBx to promote viral replication, and they further suggest that the two proteins must interact through their natural binding sites to exert their stimulatory activity.

To examine whether HBx produced from the viral template requires endogenous DDB1 for activity, we assessed the consequences for HBV replication of inhibiting DDB1 gene expression by RNA interference (RNAi), a strategy that we previously found to work efficiently (20). Because HepG2 cells transfect poorly, downregulation of DDB1 was accomplished by infecting the cells with a lentivirus vector directing the synthesis of siRNA against DDB1 from the H1-RNA gene promoter (20). Four days after infection, the cells were transfected with wild-type or HBx mutant HBV constructs, and core particle-associated HBV DNA was analyzed 3 days later as before. Figure 2 shows that RNAi-mediated inhibition of DDB1 leads to a pronounced decrease in wild-type HBV replication but has no effect on replication of the HBx mutant genome (Fig. 2A, compare lane 1 to lane 3 and lane 2 to lane 4). This is fully consistent with HBx also acting in association with DDB1 when expressed from its own viral genome.

The HBx-DDB1 complex functions in the nucleus to enhance HBV mRNA levels. HBx localizes to, and has reported activities in, both the cytoplasm and the nucleus of the cell (see the introduction). To determine in which cellular compartment HBx functions together with DDB1 to stimulate HBV replication, we made use of GFP-tagged HBx variants bearing, respectively, a heterologous nuclear localization signal (NLS) or nuclear export signal (NES) at the amino terminus (3). Previous work has shown that the NLS-containing GFP-HBx variant accumulates mostly in the nucleus whereas NESGFP-HBx is largely excluded from the nucleus, and only the former derivative was found to retain cytotoxic activities (3). Figure 3A reveals that GFP-HBx exhibits the same wild-type ability to promote HBV replication when targeted to the nucleus as it does when expressed in its original form, whereas it displays

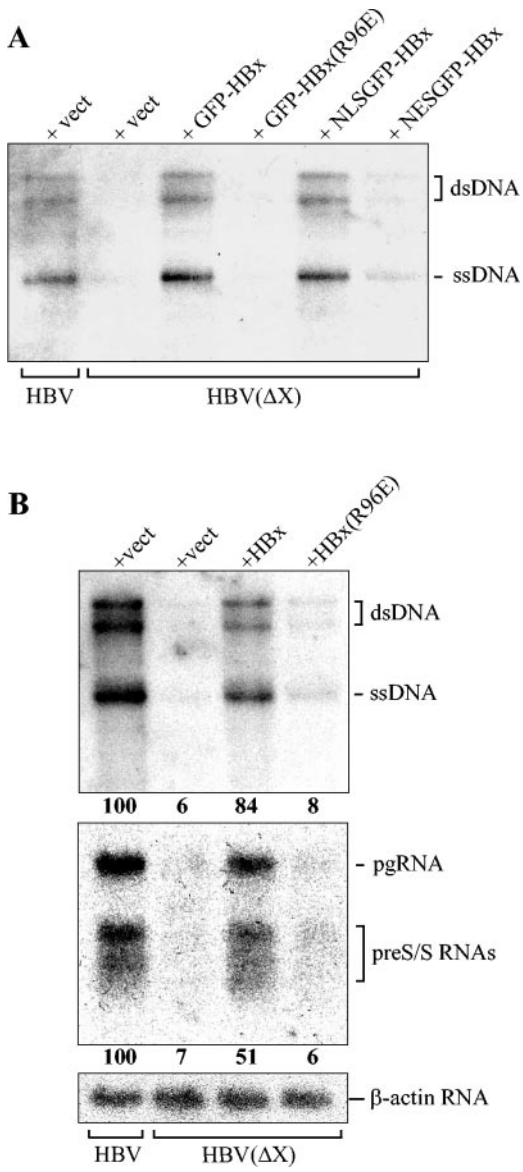

FIG. 3. HBx functions in the nucleus and acts mostly on HBV RNA levels. (A) HepG2 cells were cotransfected with wild-type HBV or HBV(ΔX) genomic DNA and equal amounts of empty vector (vect) or the indicated GFP-HBx fusion proteins and variants bearing, respectively, a heterologous nuclear localization signal (NLSGFP-HBx) or nuclear export signal (NESGFP-HBx) at the amino terminus. Transfection efficiencies were comparable as assessed by cotransfection of a GFP gene and FACS analysis (data not shown). The amount of core particle-associated HBV DNA replicative intermediates was assessed 3 days after transfection as described in the legend to Fig. 1. The single-stranded (ssDNA) and double-stranded (dsDNA) HBV DNA replicative forms are indicated on the right. (B) Quantitative analysis of viral genomic DNA and RNA levels. HepG2 cells were cotransfected with the indicated HBV genomic constructs and HBx expression plasmids. Transfection efficiencies were similar based on cotransfection of a GFP gene (data not shown). Three days after transfection, equal numbers of cells were collected, and one-half of each culture was used to purify and analyze core particle-associated HBV DNA as before (upper blot). Total cellular RNA was extracted from the other half of the culture and used to determine HBV RNA levels by Northern blot analysis (lower blot). Hybridization was performed with a ^{32}P-labeled full-length HBV probe. The positions of the HBV RNA species are indicated on the right (pgRNA, pregenomic RNA; preS/S RNAs, HBV surface antigen mRNAs). The small mRNA encoding HBx has migrated out of the gel. Quantitation was performed by phosphorimager analysis. The values indicated below each lane are relative to the levels of DNA and RNA seen with wild-type HBV, which were assigned a value of 100. Intensity values from Northern blotting of HBV RNA were normalized to β-actin. Representative results from one of three independent transfection experiments are shown.

much reduced activity when excluded from the nucleus. We therefore conclude that GFP-HBx exerts most of its stimulatory activity by forming a complex with DDB1 in the nucleus, and we suggest that the same is true when HBx is expressed in its native context from the HBV genome.

The requirement for HBx to reside in the nuclear compartment for activity suggests that the protein might promote HBV replication by regulating the abundance of HBV transcripts. To address this issue, we directly compared the effects of HBx on viral genomic DNA and RNA levels. HepG2 cells were transfected either with wild-type HBV or with the HBx mutant genome alone or together with an HBx expression plasmid. Viral replication was assayed, as before, by Southern blot hybridization of viral DNA extracted from purified cytoplasmic core particles (Fig. 3B, upper blot), whereas the amount of viral RNA was determined by Northern blot analysis of total cellular RNA isolated 3 days after transfection (Fig. 3B, lower blot). Figure 3B shows that the HBx-mediated increase in the level of HBV DNA replicative intermediates is associated with a similar increase in the levels of viral mRNAs, regardless of whether the HBx protein is expressed from the HBV genome itself or from a recombinant vector. Quantitation by phosphorimager analysis revealed that the magnitude of this increase in mRNA levels is sufficient to explain HBx stimulation of viral replication (Fig. 3B). The same results were obtained when different amounts of HBV genomic DNA were used for transfection and when the genomic DNA-to-HBx vector ratio was changed (data not shown). Taken together, these results demonstrate that HBx promotes HBV replication by acting predominantly in the nucleus to enhance the amount of HBV transcripts by a mechanism that involves its binding to DDB1.

The HBx-DDB1 complex promotes HBV replication through a pathway distinct from that leading to cell death. The results presented thus far indicate that HBx displays identical requirements with respect to DDB1 binding in order to promote HBV replication and interfere with cell viability, raising the possibility that the two activities are the same. However, the following experiments argue that this is not the case. Thus, in contrast to what is observed in HepG2 cells and in accord with previous studies (25, 41, 43, 44), HBx has no effect on HBV replication in the human hepatoma Huh-7 cell line; indeed, the HBx mutant HBV genome replicates at levels similar to wild type in these cells (Fig. 4A). Yet, HBx exhibits comparable cytotoxic activities, as judged from a colony formation assay in HepG2 cells and in Huh-7 cells, which both express DDB1 (Fig. 4B, bottom), and it does so whether produced from a recombinant plasmid (Fig. 4C) or from the viral genome (Fig. 4D). As expected, the DDB1-binding-defective HBx(R96E) mutant lacks such activity (Fig. 4C). Although we could not detect HBx expressed in its native form from the viral genome or from a recombinant vector, the use of a GFP-HBx variant revealed that the protein is actually expressed at higher levels in Huh-7 cells than in HepG2 cells (Fig. 4B, top), indicating that the lack of stimulatory activity of HBx in Huh-7

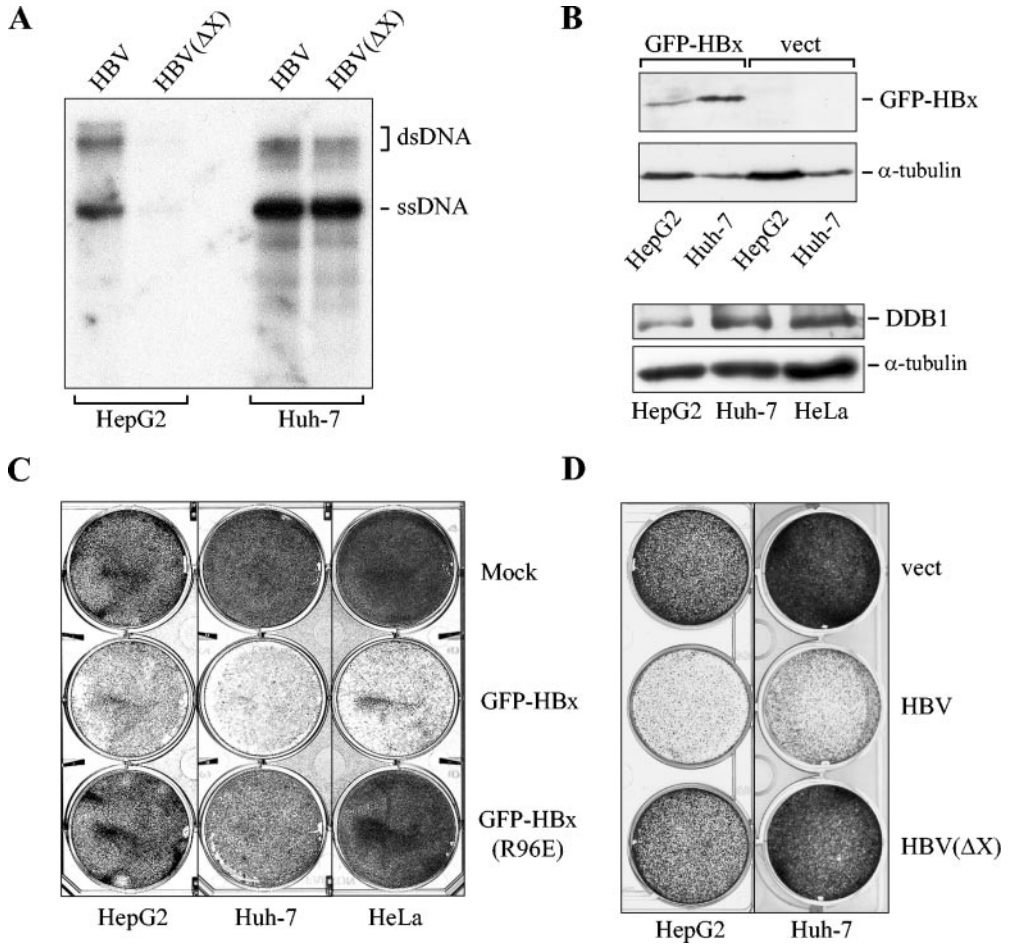

FIG. 4. HBx promotes HBV replication and induces cell death through distinct DDB1-dependent pathways. (A) Human hepatoma HepG2 and Huh-7 cells were transfected with wild-type HBV or HBV(ΔX) genomic DNA together with a GFP gene to assess transfection efficiencies by FACS analysis. The amount of core particle-associated HBV DNA replicative intermediates was assessed by Southern blot analysis 3 days after transfection as described in the legend to Fig. 1. Transfection efficiencies were similar for the two genomic constructs in each cell line, but important differences were noticed between the two cell lines (~5% in HepG2 cells versus ~20% in Huh-7 cells [data not shown]). Hence, the amounts of sample analyzed were corrected accordingly. One of two independent transfection experiments is shown. The single-stranded (ssDNA) and double-stranded (dsDNA) HBV DNA replicative forms are indicated on the right. (B) Western blot analysis. Whole-cell extracts prepared from HepG2 or Huh-7 cells transfected with GFP-HBx or empty vector (vect) (top) or from the indicated untransfected cell lines (bottom) were separated by gel electrophoresis. Immunoblot analyses were performed with antibodies to HBx (top), DDB1 (bottom), and, as a control for loading, α-tubulin. In the upper gel, fourfold-larger amounts of HepG2 protein extract were loaded on the gel to correct for transfection efficiencies. (C) Clonogenic cell survival assay. HeLa, Huh-7, and HepG2 cells were either mock transduced (Mock) or transduced with lentivirus vectors expressing the indicated GFP-HBx fusion proteins. Transduction efficiencies were comparable, as assessed by FACS analysis (data not shown). Cells were then seeded at appropriate dilutions in six-well culture dishes. After 16 days of undisturbed growth at 37°C, the surviving cells were fixed and stained with crystal violet. (D) Huh-7 and HepG2 cells were transfected with a GFP-expressing plasmid bearing a hygromycin resistance gene either alone (vect) or together with equal amounts of the indicated HBV genomic DNA. The transfected cells were seeded at appropriate dilutions in a six-well culture dish and cultured in medium containing hygromycin. Drug-resistant colonies were fixed and stained with crystal violet 20 (HepG2) or 15 (Huh-7) days after transfection. Note that the HBV replication assay presented in panel A was performed 3 days after transfection, at which time the HBx-expressing HepG2 and Huh-7 cells do not show any of the obvious changes in morphology that typically precede HBx-mediated cell death (data not shown).

cells is unlikely to be due to protein instability. These results lead us to propose that HBx stimulates HBV replication through its association with DDB1 via a pathway that differs at some point from that leading to cell death.

DISCUSSION

The HBx protein has been reported to exhibit a variety of different activities in tissue culture cells, including induction of cell death and stimulation of HBV replication. Previous work established the functional importance of an interaction between HBx and the cellular protein DDB1 in HBx-mediated cell death (20, 21, 30). In the present study, we show that this interaction is also critical for HBx to promote HBV genome replication in human hepatoma cells. This stimulatory function of HBx shows the same requirements with respect to DDB1 binding as its previously reported effect on cell viability; it needs HBx and DDB1 to interact through their natural binding regions and to form a complex in the nuclear compartment of

the cell. However, HBx exhibits DDB1-dependent cytotoxic properties in both HepG2 and Huh-7 hepatoma cells, whereas it enhances HBV replication only in HepG2 cells, suggesting that the two activities are distinct. The finding that HBx performs these activities when expressed at more physiological levels from its own viral genome rather than from a recombinant vector suggests that they are potentially relevant to HBV infection and/or associated liver carcinogenesis.

Recent studies (4, 17) reported that HBx functions in the cytoplasm to stimulate HBV DNA replication by enhancing the reverse transcription activity of the viral polymerase while having little effect on viral mRNA steady-state levels. In marked contrast, we found that HBx acts predominantly, if not exclusively, to increase viral mRNA levels regardless of whether it is expressed from its own viral genome or from a recombinant vector. Furthermore, the use of HBx variants engineered to accumulate in either the cytoplasm or the nucleus suggests that HBx performs this activity by a mechanism that requires its nuclear location. These contrasting results are not easy to explain, since the same HBV genomic constructs and recipient HepG2 cell line were used in both studies. One possibility is that HBx can promote HBV replication by performing distinct activities, depending on the cell culture conditions. It would be interesting to see if the effect of HBx on viral polymerase does also require its interaction with DDB1.

Although we did not specifically address this issue, HBx is likely to increase the steady-state levels of HBV mRNAs by stimulating viral transcription (7, 25, 40, 42). Indeed, the protein is widely recognized to display transactivation activity (26), and evidence has been presented that at certain promoters this activity requires an interaction of HBx with DDB1 (30, 36). Although HBx is believed to function predominantly in the cytoplasm to stimulate various signal transduction pathways through its association with mitochondria (1, 35), our finding that HBx exerts its effect in the nucleus is more consistent with the HBx-DDB1 complex promoting viral replication by another mechanism. In agreement with such a possibility, previous studies have shown that HBx increases expression of an HBV enhancer I reporter construct through a pathway that involves a nuclear function of HBx (12). HBx and DDB1 are therefore more likely to function directly at the HBV promoter level. DDB1 has been reported to bind to and function as a transcription factor at the apolipoprotein B (*apoB*) (18) and double-stranded RNA-dependent protein kinase (PKR) promoters (10), whereas HBx does not bind to DNA directly but exhibits transactivation properties when fused to a heterologous DNA-binding protein (28, 33). It is possible, therefore, that DDB1 binds to some HBV regulatory sequence with HBx acting as a coactivator. Alternatively, HBx may associate with HBV enhancer-bound transcription factors, such as CREB, ATF-2 (23), or AP-2 (28), and through its interaction with DDB1 recruits either STAGA (24) or a DDB1-containing E3 ubiquitin ligase complex (15) to modify chromatin components or transcription factors.

Intriguingly, although a direct comparison of the levels of viral replication in HepG2 and Huh-7 cells is made difficult due to large differences in transfection efficiencies, HBV appears to replicate at comparable if not higher levels in Huh-7 cells, in which HBx has no stimulatory effect, than wild-type HBV does in HepG2 cells. These results suggest that HBx may act by relieving the effect of an inhibitory activity. It has been reported that p53, which is functional in HepG2 cells and mutated in Huh-7 cells (5), can repress HBV transcription by binding specifically to the HBV enhancer I and that HBx can prevent p53 from exerting this repressive effect (11), perhaps by interacting directly with p53 (19). However, under our experimental conditions, we did not detect any effect on replication of the wild-type or HBx mutant HBV genome upon siRNA-mediated downregulation of endogenous p53 expression in HepG2 cells or cotransfection of a wild-type p53 expression construct in Huh-7 cells (data not shown). Hence, the mechanism through which HBx mediates its activity in association with DDB1 remains to be established.

Whether the DDB1-dependent stimulatory activity of HBx described in the present work is related to the essential function of HBx during natural viral infection remains to be determined. However, evidence has been presented that mutations in the woodchuck hepatitis virus X gene that variably affect binding of WHx to DDB1 in vitro either delay or abolish productive virus infection in woodchucks, consistent with this interaction being functionally important (29). Surprisingly, in these studies, no correlation was observed when the WHx mutant virus constructs were tested for replication in HepG2 cells (29), in which HBx-mediated HBV replication involves DDB1. However, we note that HBV replication shows a strong dependence on HBx only under certain experimental conditions, for reasons that are not clear. Since HBx fully substitutes for WHx in activating woodchuck hepatitis virus replication (17), it is more likely that the WHx mutant constructs were tested under conditions where replication was mostly independent of WHx.

The finding that, when expressed in its most native context from the viral genome, HBx affects the viability of hepatoma cell lines is rather unexpected, since HBV is a noncytopathic virus. An interesting possibility is that HBx exhibits deleterious activities only in actively dividing cells but not in quiescent cells, and experiments aimed at addressing this issue are ongoing. Following this hypothesis, HBx could promote HBV replication without altering cell viability at the time of infection, when most hepatocytes are in a quiescent state. However, HBx might become cytotoxic at later stages of the infection, and thereby possibly contribute to liver cancer development, when hepatocytes divide to replace those destroyed by the host immune response. Since both activities require the binding of HBx to DDB1, this HBV-host protein interaction may represent a promising new target for therapeutic intervention.

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