Hepatitis B Virus X Protein Associated with UV-DDB1 Induces Cell Death in the Nucleus and Is Functionally Antagonized by UV-DDB2*

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The hepatitis B virus X protein (HBx) is essential for viral infection and strongly interferes with cell growth and viability in culture. These activities involve interaction of HBx with the DDB1 subunit of UV-damaged DNA-binding factor UV-DDB. UV-DDB consists of DDB1 and a DDB2 subunit that mediates nuclear import and has recognized functions in DNA repair and E2F1-mediated transcription. Here we show that HBx retains DDB1-binding-dependent cytotoxic activities when engineered to accumulate in the nucleus but not when excluded from the nucleus. Nuclear localization of HBx does not require binding to DDB1 and remains unaffected by ectopically expressed UV-DDB subunits, indicating that HBx reaches the nuclear compartment independently of UV-DDB. Unexpectedly, HBx appears to largely exist in association with DDB1 and is in direct competition with DDB2 for binding to DDB1. Hence, HBx-mediated cell death can be relieved by increased levels of DDB2, an effect that is not observed with a naturally occurring mutant of DDB2 that lacks DDB1-binding activity. These findings indicate that HBx acts through a pathway that involves a DDB2-independent nuclear function of DDB1 and that this activity will depend on the relative concentration of DDB1 and DDB2 in cells.

Hepatitis B virus (HBV)† belongs to the family Hepadnaviridae and causes both acute and chronic infections of the liver. Chronic infection with HBV is associated with a high risk of developing liver cancer. HBV encodes a small regulatory protein called HBx that is well conserved among all mammalian hepatitis viruses. HBx is essential for establishing natural viral infection (1, 2) and has been specifically implicated in the development of hepatocellular carcinoma. However, the basis for HBx function in either process is not yet understood.

In cell culture, HBx localizes in both the cytoplasm and the nucleus, and it behaves as a multifunctional protein affecting transcription, cell cycle control, cell growth, and apoptotic cell death (for reviews, see Refs. 3 and 4). The protein is believed to perform many of its activities in the cytoplasmic compartment where it stimulates various signal transduction pathways (5–8), interferes with proteasome (9, 10) and mitochondrial (11, 12) functions, and stimulates HBV DNA replication by triggering the release of Ca2+ into the cytosol (13). However, HBx also has reported nuclear functions. For example, a nuclear location has been shown to be essential for the protein to stimulate transcription of the HBV enhancer I (14). HBx is thought to mediate these various activities through interactions with cellular factors. Indeed, a number of potential cytoplasmic and nuclear targets have been reported to bind HBx, including the DDB1 subunit of the UV-damaged DNA-binding factor (UV-DDB) (15, 16). The functional significance of most of these interactions remains, however, largely elusive.

The binding of HBx to DDB1 is essential for HBx to activate transcription (17) and to induce cell death in culture (17, 18). Binding to DDB1 is a conserved feature among the mammalian X proteins (16), and evidence has been presented that this interaction is critical for efficient hepatitis B virus infection in woodchuck (19). DDB1 is a 127-kDa protein that associates with DDB2, a cell cycle-regulated, UV-inducible 48-kDa protein that transports DDB1 from the cytoplasm to the nucleus (20–23). DDB1 and DDB2 form the UV-DDB complex that exhibits high binding affinity for UV-damaged DNA (24–29). UV-DDB has been implicated in nucleotide excision repair (30–33) and is deficient in some cancer-prone disease xeroderma pigmentosum group E patients due to mutations of the DDB2 gene (29). No mutations in DDB1 have been reported. Interestingly, a role for UV-DDB other than in DNA repair has also been suggested. UV-DDB functionally interacts with the cell cycle transcription factor E2F1 to stimulate transcription of E2F1-regulated genes, suggesting that it plays a role in the cell cycle (23). Although these two known activities depend upon both UV-DDB subunits, the high evolutionary conservation of DDB1, but not of DDB2, suggests that DDB1 might also carry out important functions independently of DDB2 (34, 35).

The mechanism whereby HBx interferes with cell viability upon binding to DDB1 is not understood, nor has the contribution of the cytoplasmic and nuclear fraction of HBx been clearly defined. Several observations argue against HBx exerting its effect simply by sequestering DDB1, and thereby preventing its normal function. Thus, overexpression of DDB1 does not relieve, and under certain experimental settings even enhances, HBx toxicity (17, 18). Moreover, Sitterlin et al. (17) describe a mutant of woodchuck hepatitis virus X protein that exhibits increased DDB1-binding activity yet lacks cytotoxic properties. In a converse experiment we found that a DDB1 binding-defective HBx mutant that cannot interact with endogenous DDB1 regains cytotoxic activities when ectopically fused to DDB1 (18). HBx therefore may act by forcing DDB1 into its physiological function or by conferring new activities to the DDB1 protein.
In the present study we demonstrate that HBx induces cell death by acting in association with DDB1 in the nuclear compartment. We also show that HBx and UV-DDB translocate into the nucleus independently, and that HBx and DDB2 compete for interaction with DDB1. Thus, HBx-mediated toxicity can be relieved by increasing the cellular concentration of the DDB2 protein. These findings indicate that HBx acts through a pathway that involves a DDB2-independent nuclear function of DDB1 and that it will do so depending on the relative concentration of DDB1 and DDB2 in the cell.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—All recombinant DNA work was done according to standard procedures. Details of the plasmid constructions are available upon request.

The mammalian expression vectors used in this study were pBJ3, EBS-PL, REBOB-PL, and pHREOS. Plasmid pH3 in which the simian virus 40 (SV40) early enhancer/promoter drives expression was kindly provided by Bruno Amati, DNAx Research Institute, Palo Alto, CA. The episomal Epstein-Barr virus-based expression vector EBS-PL has been described previously (36). It carries a hygromycin resistance-conferring gene and permits expression from the strong SRα promoters (37). The episomal vector REBOB-PL is a modified version of EBO-76PL (18). It contains the same SV40 early promoter but carries a kanamycin resistance gene isolated from pUC4K (a generous gift from Dominique Belin, University of Geneva Medical School, Geneva, Switzerland), which replaces the original β-lactamase gene, and a blasticidin resistance gene isolated from pCDNA6/V5-His (Invitrogen) as a selectable marker for mammalian cells in place of the hygromycin resistance gene. Plasmid pSRαS was constructed from pCI-neo (Promega) by replacing the original cytomegalovirus promoter and β-globin/IgG chimeric intron by the SRα promoters and the SV40 late gene (16S) splice junction isolated from pCDL-SRα296 (37). GFP, produced either from pEGFP-C1 (CLONTECH) or from the GFP open reading frame of pEGFP-N1 (CLONTECH) cloned into pSRαS, was used as a control to assess for transfection efficiency.

Wild-type HBx and the point mutants HBx(S96E) and HBx(S189F) expressed as native proteins or as N-terminal GFP fusions were described previously (18). The NLS motif derived from simian virus 40 large T antigen was linked in-frame to the N terminus of HBx and GFP-HBx. This was done by the cloning of a double-stranded oligonucleotide encoding the amino acid sequence MKKKKRKA (the NLS sequence is underlined), thus generating a fusion protein (38) to HBx and GFP-HBx using oligonucleotides encoding the amino acid sequence MELALKLGLDINKA (the NLS sequence is underlined). DNA segments encoding full-length DDB1 (34), DDB2 (39), and XIP (40) were constructed by inserting three copies of a double-stranded oligonucleotide encoding peptide MEQKLISEEDLHMH (the myc epitope tag is underlined) in front of the open reading frame. GFP-DDB1 was constructed by fusing in-frame the GFP coding region excised out of PCR fragments were verified by sequence analysis. GFP-DDB1 and was constructed by inserting three copies of a double-stranded oligonucleotide encoding peptide MEQKLISEEDLHMH (the myc epitope tag is underlined) in front of the open reading frame.

Cell Cultures and Transfections—HeLa and HepG2 cells were transfected using the FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. When not made as a fusion protein, an expression plasmid for GFP was cotransfected (10% of total DNA). At 24 h post-transfection, cells were trypsinized and a fraction (usually 1/3) was scanned by FACScan for GFP fluorescence to assess for transfection efficiencies; transfection efficiencies were generally 50–80% with variations of less than 10% within any single experiment.

**Fluorescence Microscopy**—Cells were grown on coverslips and transfected. 1–2 days after transfection, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline and stained with 5 μg/ml Hoechst 33342 (Sigma), and coverslips were mounted onto glass slides using Mowiol 4-88 (Calbiochem). Cells were viewed in a Zeiss Axiopt fluorescence microscope equipped with a Plan-Neofluar 40×/1.30 oil objective. Pictures were acquired with an AxioCam color charge-coupled device camera (Zeiss).

**Immunoprecipitation and Western Blotting**—Cell extracts for Western blot analysis were prepared as described previously (18). The immunoprecipitation experiments presented in Fig. 3 were performed using whole-cell extracts prepared 24 h after transfection. A total of 2 × 10⁶ HeLa cells was lysed on the plate in 1.5 ml of Nonidet P-40–containing lysis buffer (0.1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.6 mM phenylmethylsulfonyl fluoride supplemented with a complete protease inhibitor mixture from Roche Molecular Biochemicals) for 20 min on ice with gentle agitation. Plates were then scraped, and the crude lysates were cleared by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatants were collected and adjusted to a final concentration of 20%, and the samples were frozen in liquid nitrogen prior to storage at −70 °C. HA-DDB1 was immunoprecipitated from 250 μg of whole-cell extracts by incubation with 70 μl of anti-HA affinity matrix (Roche Molecular Biochemicals) in a final volume adjusted to 700 μl with lysis buffer. Myc-DDB2 was immunoprecipitated from 50 μg of whole-cell extract in a final volume of 500 μl of lysis buffer containing 10 μg of myc antibody (anti-myc affinity matrix (Roche Molecular Biochemicals)) coupled to 20 μg of protein G-agarose. GFP-HBx was immunoprecipitated from 50 μg of whole-cell extract using one-fourth of a mixture containing 50 μg of rabbit polyclonal anti-GFP antibody (Abcam ab290) coupled to 40 μl of protein A-agarose CL-4B. After incubation for 3 h at 4 °C with constant rotation on a rocker, the beads were washed twice in lysis buffer. The beads were then resuspended and boiled in Laemmli buffer. One-half of the supernatant was analyzed by SDS-PAGE (9% polyacrylamide). After separation, the proteins on the gels were transferred to Immobilon P (Millipore) and subjected to immunoblotting as described previously (18). Membranes were probed with anti-myc monoclonal antibody (mAb) 9E10, anti-HA mAb 16B12 (BAbCO), anti-GFP mAb (mixture of clones 7.1 and 13.1 from Roche Molecular Biochemicals), and polyclonal rabbit anti-CIITA serum (36). Binding of primary antibody was detected with anti-rabbit or anti-mouse immunoglobulin, horseradish peroxidase-conjugated (Bio-Rad Laboratories), and membranes were developed with ECL reagents (Amersham Biosciences). Blotted proteins were visualized with Lumi-Light or Lumi-LightPLUS blotting reagents (Roche Molecular Biochemicals).

**RESULTS**

**Nuclear but Not Cytoplasmic HBx Induces Cell Death**—Consistent with previous studies, we found that an HBx construct bearing an N-terminal enhanced green fluorescent protein (GFP) localizes in both the cytoplasm and the nucleus of HeLa cells, with a substantial fraction residing in the nucleus (Fig. 1A). A similar subcellular distribution of GFP-HBx was observed in the human hepatoblastoma cell line HepG2 (data not shown). To determine if cellular compartment HBx manifests cytotoxic activities, we engineered HBx and GFP-HBx derivatives containing either a nuclear localization signal (NLS) or a nuclear export signal (NES) at the N terminus. As
shown in Fig. 1A, the presence of an NLS causes efficient nuclear accumulation of GFP-HBx, whereas an NES virtually excludes it from the nucleus. We then examined the proteins for their ability to suppress cell colony formation, a property documented to reflect HBx potential to induce cell death by apoptosis (41–43). Remarkably, Fig. 1B reveals that HBx targeted to the nucleus is able to inhibit colony formation to the same extent as the native protein, whereas it displays no obvious activity when excluded from the nucleus. This points to the nuclear fraction of wild-type HBx as being responsible for mediating cell death. We also assessed whether nuclear targeting by fusion to an NLS would restore activity to the DDB1 binding-defective HBx(R96E) point mutant. This is not the case (Fig. 1B), nor does nuclear targeting confer cytotoxic properties to DDB1 in the absence of HBx (data not shown). These results indicate that the need for an interaction between HBx and DDB1 for HBx cytotoxicity is not solely to allow one protein to promote nuclear import of the other. We thus conclude that HBx must form a complex with DDB1 in the nucleus to exert its deleterious activities.

HBx Enters the Nucleus Independently of DDB—The finding that HBx acts in association with DDB1 in the nucleus prompted us to investigate whether overexpression of DDB1, which has been implicated in the nuclear localization of HBx (17), or DDB2, which promotes nuclear import of DDB1 (23), would induce a change in the cellular distribution of HBx. We therefore transfected GFP-HBx alone or with either DDB1 or DDB2 into HeLa cells under conditions where the UV-DDB subunits are produced from an expression vector carrying a strong promoter. As can be seen in the upper panel of Fig. 2A, the nucleocytoplasmic distribution of GFP-HBx is not noticeably affected by cotransfection with either DDB1 or DDB2. Yet
under the same experimental conditions DDB2 efficiently increases accumulation of an N-terminal GFP variant of DDB1 in the nucleus (Fig. 2A, lower panel). These results suggest that nuclear compartmentalization of HBx does not depend on DDB.

If so, one would predict that HBx derivatives compromised for interaction with DDB1 should remain fully capable of nuclear entry. To determine if this is indeed the case, we examined the subcellular distribution of two HBx point mutants, HBx(R96E) and HBx(L98F), that are selectively defective for DDB1 binding (18). Fig. 2B shows that the mutants expressed as fusions to GFP exhibit the same preferential nuclear localization as the wild-type protein, with GFP-HBx(L98F) actually being mostly nuclear, perhaps as a result of the mutation also impairing the reported NES function of HBx (44). Thus, nuclear accumulation of HBx does not correlate with its binding to DDB1. Taken together, these results support the notion that HBx and DDB1 must from a complex in the nucleus to mediate cytotoxic activities, yet translocate into the nuclear compartment independently.

**Binding of HBx and DDB2 to DDB1 Is Mutually Exclusive**—The two known activities in which DDB1 has been implicated, damaged DNA binding and stimulation of E2F1-activated transcription, both require its association with DDB2 in the nucleus (23, 27, 29, 30). This may indicate that HBx functions in a complex with the DDB1-DDB2 dimer. However, we failed to obtain any evidence that HBx and DDB2 can form a ternary complex with DDB1 (data not shown). This led us to examine whether HBx and DDB2 might instead bind to DDB1 in a mutually exclusive fashion. To test this possibility, we performed coimmunoprecipitation experiments to assess whether HBx and DDB2 would interfere with each other for binding to limiting amounts of DDB1. For this purpose, we constructed N-terminally epitope-tagged HA-DDB1 and myc-DDB2 proteins, which behave like wild-type (data not shown). Plasmids expressing HA-DDB1, myc-DDB2, and GFP-HBx were transiently transfected into HeLa cells, either in pairwise combinations or all three together. In these experiments we transfected 5-fold less HA-DDB1 to ensure that myc-DDB2 and GFP-HBx are produced in excess over HA-DDB1. Whole-cell extracts were prepared, HA-DDB1 was immunoprecipitated with an anti-HA antibody, and proteins present in the extract before or after immunoprecipitation were detected by Western blot analysis. As shown in Fig. 3A, the proteins were expressed at comparable levels in all the transfected cells (inputs panel in Fig. 3A, compare lanes 1 to 4), and equal amounts of HA-DDB1 are precipitated from the relevant lysates (IP panel in Fig. 3A). Coimmunoprecipitated myc-DDB2 and GFP-HBx are detected with extracts from HA-DDB1-expressing cells but not from control cells (CO-IP panel in Fig. 3A). Importantly, however, the amount of myc-DDB2 and GFP-HBx that coimmunoprecipitates with HA-DDB1 is modestly but consistently reduced when both proteins are expressed along with HA-DDB1 (CO-IP panel in Fig. 3A, compare lane 3 with lanes 1 and 2). These results are consistent with HBx and DDB2 competing for binding to DDB1.

We also performed the reciprocal coimmunoprecipitation experiments to evaluate the amount of HA-DDB1 protein that coimmunoprecipitates with myc-DDB2 and GFP-HBx from these extracts. One would expect that the binding of one protein to DDB1 would limit the amount of DDB1 available to interact with the other. Fig. 3B and C show that this is indeed the case. The HA-DDB1 protein is detected in all the relevant myc-DDB2 and GFP-HBx immunoprecipitates, but the amount recovered from extracts containing both proteins is reduced compared with the amount observed with extracts containing either protein alone (CO-IP panels in Fig. 3, B and C, compare lane 3 with lanes 1 and 2). In these experiments, myc-DDB2 and GFP-HBx did not detectably coprecipitate (data not shown).

**FIG. 3.** HBx and DDB2 compete for binding to limiting amounts of DDB1. A, coimmunoprecipitation experiments with extracts from transiently transfected HeLa cells. Equal amounts of plasmids expressing N-terminally epitope-tagged myc-DDB2 and GFP-HBx proteins, and 5-fold less plasmid-expressing HA-DDB1 were transfected in pairwise combinations or all three together. All constructs were expressed in pSRoS. Total plasmid DNA was kept equal by adding empty plasmid DNA. Whole-cell extracts were prepared 1 day after transfection and subjected to immunoprecipitation with a monoclonal antibody against the HA epitope. The immunoprecipitates were separated by SDS-PAGE and analyzed by Western blot assays for the presence of HA-DDB1 with an anti-HA antibody (IP). The presence of coimmunoprecipitated myc-DDB2 and GFP-HBx was detected using, respectively, anti-myc and anti-GFP monoclonal antibodies (CO-IP). The inputs panel shows 1/20 of the cell extract used in the immunoprecipitations to assess for comparable protein levels. Reduced amounts of myc-DDB2 and GFP-HBx in the precipitates from cells expressing both proteins along with HA-DDB1 were observed with extracts from two independent transfection experiments. B and C, the same extracts were used to compare among the transfected cells the amount of HA-DDB1 that coimmunoprecipitates with myc-DDB2 (B) or with GFP-HBx (C). In these experiments, myc-DDB2 and GFP-HBx did not detectably coprecipitate (data not shown).
HBx/UV-DDB2 Act in the Nucleus Independently of UV-DDB2

The relative concentration of DDB1 and DDB2 determines cellular HBx protein levels. GFP-HBx, the DDB1 binding-defective GFP-HBx(R96E) point mutant and HA-DDB1 (upper and middle panels), or the unrelated major histocompatibility class II transcriptional coactivator CIITA-D5 (lower panel), were individually cotransfected in HeLa cells together with plasmids expressing the indicated proteins. All constructs were expressed in EBS-PL, except HA-DDB1 that was produced from pSRoS. Protein accumulation was analyzed 3 days post-transfection by FACS and by Western blot analyses. The FACS analysis shows the expression levels of GFP-HBx and GFP-HBx(R96E) when cotransfected with the empty vector EBS-PL (filled profile) or with plasmids expressing the proteins indicated on top (open profile). XIP is a cellular protein that has been reported to interact with HBx (40). Nontransfected cells (nt).

The Stoichiometry of DDB2 Relative to DDB1 Determines HBx Protein Levels in Cells—If HBx and DDB2 were indeed competing for binding to DDB1, one would predict that the fraction of DDB1 available for interaction with HBx would depend on the relative concentration of DDB1 and DDB2 in the cell. If so, this would profoundly impact on the amount of HBx present in the cell as HBx and DDB1 strongly stabilize each other upon binding (18). To test this hypothesis, we examined the consequence of expressing DDB1 and DDB2 in excess over the endogenous proteins on the cellular level of GFP-HBx. It should be noted that these experiments were performed using cell extracts prepared 3 days after transfection, because no differences in protein levels could be detected at 1-day post-transfection (inputs panel in Fig. 3A). As reported previously (18), cotransfection of DDB1 and GFP-HBx in HeLa cells leads to accumulation of GFP-HBx, and this effect is detectable both by fluorescence-activated cell sorting (FACS) and by Western blot analyses (upper and middle panels in Fig. 4). In marked contrast to DDB1, cotransfected DDB2 reduces the amount of GFP-HBx to barely detectable levels (upper and middle panels in Fig. 4). This is unlikely to result from DDB2 destabilizing endogenous DDB1, because DDB2 actually increases the level of a HA-tagged DDB1 variant in a control experiment (middle right panel in Fig. 4). The effects on HBx are specific for DDB1 and DDB2; no changes in GFP-HBx protein levels are observed upon cotransfection of HBx or XIP, another cellular protein documented to interact with HBx (40) (upper and middle panels in Fig. 4). Furthermore, they depend on HBx interacting with DDB1, because the GFP-HBx(R96E) point mutant, which is defective for DDB1 binding, remains unaffected (Fig. 4, upper panel). Because HBx has reported DDB1 binding-dependent transactivation activities (16), we considered the possibility that ectopically expressed UV-DDB subunits might influence GFP-HBx protein levels indirectly, by regulating its transactivation potential on its own promoter. However, the lower panel in Fig. 4 shows that no significant difference in the expression level of an unrelated protein produced from the same vector is detected upon cotransfection of these proteins alone or in combination. These results argue that a physical interaction with DDB1 is the major mechanism whereby HBx is stabilized within the cell, and they indicate that DDB2 alters HBx protein levels indirectly as a result of displacing it from the endogenous DDB1 protein.

Increased Levels of DDB2 Relieve HBx-mediated Cell Death—The finding that HBx and DDB2 bind to DDB1 in a mutually exclusive manner has important functional implications: an increase in intracellular concentration of the DDB2 subunit should preclude HBx interaction with DDB1 and thereby prevent it from inducing cell death. To test this hypothesis, we investigated whether cotransfection of the DDB2 gene could rescue the HBx transfecants as measured by the colony formation assay. As reported previously (17, 18), coexpression of DDB1 does not relieve, and in some experiments may even enhance, suppression of colony formation by HBx (left panel in Fig. 5A, and upper panel in Fig. 5C). This latter effect may either result from DDB1 weakly interfering with normal cell growth when expressed at high levels (45), or reflect HBx stabilization by coexpressed DDB1 (Fig. 4). Remarkably, coexpression of DDB2 largely overcomes HBx-dependent cell death (left panel in Fig. 5A). FACS analysis revealed that the surviving cells express GFP-HBx to levels comparable to those found with the functionally defective GFP-HBx(R96E) point mutant that lacks DDB1-binding activity, consistent with the DDB2 subunit effectively blocking HBx binding to endogenous DDB1 (right panel in Fig. 5A). Identical results were obtained in the human hepatoma cell line HepG2, in which HBx exhibits the same R96E mutation-sensitive, DDB2 protein concentration-dependent cytotoxic activities (Fig. 5B).

To address whether DDB2 mediates its effect through its binding to DDB1, as would be expected if the protein acts by blocking interaction of HBx with DDB1, we took advantage of two naturally occurring point mutants of DDB2 identified in xeroderma pigmentosum group E patients. Both mutants are defective for damage-specific DNA binding (22, 29). One mutant, 82TO (K244E), retains DDB1-binding activity, whereas the other, 2RO (R273H), fails to communoprecipitate with DDB1 (23). Fig. 5C shows that the DDB1 binding-proficient 82TO (K244E) mutant destabilizes GFP-HBx and exhibits reduced, but significant ability to increase survival of HBx-expressing cells, whereas the DDB1 binding-defective 2RO (R273H) mutant is ineffective in these assays. These results are fully consistent with DDB2 preventing HBx from inducing cell death by displacing it from DDB1. Given these data, we conclude that HBx promotes cell death upon forming a complex with DDB1 in the nucleus, and its ability to do so directly depends on the relative concentration of DDB1 and DDB2 in the cell.

DISCUSSION

The functional importance of an interaction between HBx and DDB1 in inducing cell death in culture has been firmly
and in relation to DDB2, the natural partner of DDB1. We demonstrate that HBx induces cell death in association with DDB1 by a mechanism that involves nuclear compartmentalization of the protein. We further show that nuclear entry of HBx occurs independently of binding to DDB1 and, unexpectedly, that HBx and DDB2 bind to DDB1 in a mutually exclusive fashion. Based on these results and on the knowledge that DDB2 acts as the nuclear transporter of DDB1 (18), an activity for which HBx cannot substitute (23), we propose the following model shown in Fig. 6. According to this model, HBx and DDB1 enter the nuclear compartment separately, DDB2 being responsible for the nuclear import of DDB1. Once inside the nucleus HBx forms a complex with DDB1, either by binding to the fraction of DDB1 that may have dissociated from DDB2 or, as suggested in the model, by displacing DDB2 from DDB1. Importantly, however, HBx does not trigger cell death by preventing DDB1 from performing its normal activities in association with DDB2 (18). Here we provide evidence that HBx also does not act by inducing degradation or harmful accumulation of free DDB2. We thus propose that it is the HBx-DDB1 complex by itself that is toxic to the cells.

The use of HBx variants engineered to accumulate in either the cytoplasm or the nucleus of the cell permitted us to demonstrate unambiguously that HBx acts in the nucleus to trigger cell death (Fig. 1). That nuclear entry of HBx occurs independently of UV-DDB stems from the following observations. First, ectopic expression of DDB1 or DDB2 under experimental conditions where DDB2 efficiently imports DDB1 into the nucleus has no noticeable effect on the intracellular distribution of HBx (Fig. 2A). Second, the two DDB1 binding-defective HBx point mutants that we identified previously exhibit preferential nuclear localization similar to the wild-type protein (Fig. 2A). The last result is in conflict with the results of Sitterlin et al. (17), who found that the nuclear localization of the woodchuck hepatitis virus X protein correlates with its DDB1-binding ability. The reason for this discrepancy is not understood but may reflect species- or cell type-specific differences or different expression levels of the viral proteins. We also failed to obtain any evidence that HBx binds to HBx independently of DDB1, as recently proposed (46), which is fully consistent with our observation that DDB2 has no effect on the subcellular distribution of HBx (Fig. 2A). Taken together, these results provide strong evidence that HBx and UV-DDB reach the nucleus independently of each other. How, then, does HBx translocate into the nucleus? The protein is a small polypeptide, and it may therefore diffuse passively through the nuclear pore complex. However, HBx preferentially compartmentalizes in the nucleus only at low expression levels (47), suggesting that its nuclear

established (17, 18), and evidence has been presented that this interaction is critical for efficient hepatitis B virus infection in woodchuck (19). In the present study we further examine the HBx-DDB1 complex with regard to its intracellular distribution
import might depend on limiting cellular proteins, such as IxBo (48).

The notion that HBx and DDB2 bind to DDB1 in a mutually exclusive fashion is supported by coimmunoprecipitation experiments (Fig. 3) and by the finding that DDB2 exhibits DDB1-binding dependent abilities to reduce HBx protein levels in the cell (Fig. 4). Because HBx is stabilized by its interaction with DDB1 (18) (Fig. 4), this last result is best explained by DDB2 displacing HBx from endogenous DDB1. As a consequence, ectopically expressed DDB2 also relieves HBx-mediated cell death (Fig. 5). These experiments provide hard evidence that interaction of HBx with DDB1 is critical for its activity. They also suggest that HBx mostly exists in association with DDB1 in the cell and, as a result, that the relative concentration of DDB1 and DDB2 may largely determine the cellular levels and activity of the viral protein. Recent studies identified DDB2 as a cell cycle-regulated protein whose level peaks at the G1/S boundary and decreases in S phase (21, 46). It is therefore conceivable that HBx can engage in productive interactions with DDB1 only at certain stages of the cell cycle.

The mechanism whereby HBx and DDB1 exert a mutual stabilizing effect remains elusive. The two proteins are reported substrates of the ubiquitin-proteasome pathway (9, 49). Therefore, it is possible that when they are in a complex they prevent each other from being targeted to the proteasomes. This effect is not specific to HBx, however, because DDB2 also stabilizes DDB1 in cotransfection experiments (Fig. 4). This is consistent with the decrease in the steady-state levels of DDB1 noticed in cells of DDB-deficient xeroderma pigmentosum group E patients (31) and with the finding that degradation of DDB1 is inhibited after UV treatment that is known to stimulate DDB2 expression (49). DDB1 also binds CUL-4A, a member of the cullin family of proteins that are components of E3 ubiquitin ligases, and CUL-4A stimulates degradation of DDB2 through the ubiquitin-proteasome pathway (21, 49, 50). DDB1 may thus serve as an adaptor to target DDB2 for proteolysis. However, neither DDB1 nor HBx have a major effect on DDB2 protein levels (data not shown), indicating that DDB1 is not limiting in the DDB2 degradation pathway and that HBx does not act by stimulating degradation of DDB2.

As yet we do not know by what mechanism HBx triggers cell death. However, our finding that the protein functions in the nucleus implies that it mediates its effect through a pathway that is not related to its various cytoplasmic activities. These include association with mitochondria (11, 12, 47), interactions with the proteasome complex (9, 10), and activation of various signal transduction pathways through which the protein is believed to exert pleiotropic transcription effects (5–8, 10, 14). HBx is also unlikely to induce harmful accumulation of DDB1 in the nuclear compartment, because the nuclear targeting of DDB1 by fusion to a NLS (data not shown) or by overexpression of DDB2 (Fig. 5) has no major effects on cell viability in the absence of HBx. The last experiment also argues against the possibility that, upon binding to DDB1, HBx provokes the release of potentially toxic amounts of free DDB2 subunits. One obvious mechanism whereby HBx might exert deleterious activities, at least when expressed at high levels, is by disrupting the DDB1-DDB2 complex due to mutually exclusive binding. Because an interaction between the UV-DDB subunits is required for UV-DDB function in stimulation of E2F1-activated transcription (23) and in DNA repair (29–32) such a scenario would readily explain the reported ability of HBx to interfere with cell cycle regulation (51) and DNA repair in certain experimental settings (52–54). However, several observations indicate that in our experiments HBx did not induce cell death by such a mechanism. Thus, in the present and past studies we found that overexpression of DDB1 does not relieve HBx-mediated cell death (18) (Fig. 5) and that DDB1 binding-defective HBx mutants that cannot interact with endogenous DDB1 nevertheless exhibit cytotoxic activities when directly fused to DDB1 (Ref. 18 and data not shown). In addition, we show here that an HBx variant engineered to relocate to the cytoplasm, and, therefore, presumably capable of interfering with DDB1-DDB2 complex formation, remains inactive in the colony formation assay (Fig. 1B). Hence we propose that the HBx-DDB1 complex by itself exerts deleterious activities. HBx must therefore perform a function that relates to a yet to be discovered DDB2-independent role of DDB1 in the nucleus. The DDB1 protein, unlike DDB2, is highly conserved among species (34, 35). Preliminary experiments indicate that in fission yeast, which lacks a DDB2 gene homologue, the DDB1 protein might carry out important functions during early mitosis.2

Evidence has been presented that an interaction between HBx and DDB1 is critical for efficient hepatitis B virus infection in woodchuck (19). However, it is not known where in the infected cell the HBx-DDB1 complex might perform its essential functions. Most studies have found HBx to localize predominantly in the cytoplasm (55–57). Yet HBx was also detected in the nuclei of a significant proportion of HBx expressing hepatocytes derived from human liver biopsies (58). Thus, the finding that the HBx-DDB1 complex acts in the nucleus could have high biological relevance. It should be stressed, however, that our results do by no means imply that HBx may not act in the cytoplasm as well. For example, a cytoplasmic location is required for HBx ability to stimulate virus DNA replication in cell culture, an activity that is likely to be significant for HBV infection (13). It will be of interest to investigate whether this and other cytoplasmic activities of HBx also depend on its association with DDB1 shown here to mediate cell death in the nucleus.

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REFERENCES
1. Chen, H. S., Kaneko, S., Girones, R., Anderson, R. W., Hornbuckle, W. E., Terr grunt, B. C., Cote, P. J., Gerin, J. L., Purrel, R. H., and Miller, R. H. (1993) J. Virol. 67, 1218–1226
2. Zoulim, F., Saputelli, J., and Seeger, C. (1994) J. Virol. 68, 2026–2030
3. Arbutnout, P., Capovilla, A., and Kew, M. (2000) J. Gastroenterol. Hepatol. 15, 357–368
4. Murakami, S. (2001) J. Gastroenterol. 36, 651–660
5. Diao, J., Khine, A. A., Sarangi, F., Hsu, E., Iorio, C., Tibbles, L. A., Woodgett, J. R., Penninger, J., and Richardsen, C. D. (2001) J. Biol. Chem. 276, 8328–8340
6. Rekule, A. S., Lauer, U., Weiss, L., Luber, B., and Hofschneider, P. H. (1993) Nature 361, 745–747
7. Kim, H., Lee, Y. W., Won, J., and Yum, Y. (2001) Biochem. Biophys. Res. Commun. 286, 886–894
8. Klein, N. P., and Schneider, R. J. (1997) Mol. Cell. Biol. 17, 6427–6436
9. Hu, Z., Zhang, Z., Dox, E., Coux, O., Goldberg, A. L., and Liang, T. J. (1999) J. Virol. 73, 7231–7240
10. Sirmia, H., Weil, R., Rosmorduc, O., Urban, S., Israel, A., Kremsdorf, D., and Brechot, C. (1998) Oncogene 16, 2051–2063
11. Rahman, Z., Huh, K. W., Lashe, R., and Siddiqui, A. (2000) J. Virol. 74, 2840–2846
12. Takada, S., Shirakata, Y., Kaneniwa, N., and Koike, K. (1999) Oncogene 18, 6965–6973
13. Bouchard, M. J., Wang, L. H., and Schneider, R. J. (2001) Science 294, 2376–2378
14. Doria, M., Klein, N., Lucito, R., and Schneider, R. J. (1995) EMBO J. 14, 4747–4757
15. Lee, T. H., Elledge, S. J., and Butel, J. S. (1995) J. Virol. 69, 1107–1114
16. Sitterlin, D., Lee, T. H., Prigent, S., Tiollais, P., Butel, J. S., and Transy, C. (1997) J. Virol. 71, 6194–6199

2 A. Krapp and V. Simanis, unpublished.
Hepatitis B Virus X Protein Associated with UV-DDB1 Induces Cell Death in the Nucleus and Is Functionally Antagonized by UV-DDB2
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