Hepatitis B Virus X Protein Modulates Apoptosis in Primary Rat Hepatocytes by Regulating both NF- κ B and the Mitochondrial Permeability Transition Pore[∇]

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The hepatitis B virus (HBV) X protein (HBx) is a multifunctional protein that regulates numerous cellular signal transduction pathways, including those that modulate apoptosis. However, different HBx-dependent effects on apoptosis have been reported; these differences are likely the consequence of the exact conditions and cell types used in a study. Many of the previously reported studies that analyzed HBx regulation of apoptosis were conducted in immortalized or transformed cells, and the alterations that have transformed or immortalized these cells likely impact apoptotic pathways. We examined the effects of HBx on apoptotic pathways in cultured primary rat hepatocytes, a biologically relevant system that mimics normal hepatocytes in the liver. We analyzed the effects of HBx on apoptosis both when HBx was expressed in the absence of other HBV proteins and in the context of HBV replication. HBx stimulation of NF- κ B inhibited the activation of apoptotic pathways in cultured primary rat hepatocytes. However, when HBx-induced activation of NF- κ B was blocked, HBx stimulated apoptosis; blocking the activity of the mitochondrial permeability transition pore inhibited HBx activation of apoptosis. These results suggest that HBx can be either proapoptotic or antiapoptotic in hepatocytes, depending on the status of NF- κ B, and confirm previous studies that link some HBx activities to modulation of the mitochondrial permeability transition pore. Overall, our studies define apoptotic pathways that are regulated by HBx in cultured primary hepatocytes and provide potential mechanisms for the development of HBV-associated liver cancer.

Worldwide, approximately 350 million individuals are chronically infected with human hepatitis B virus (HBV) (73). Chronic HBV infections are associated with the development of primary liver cancer, hepatocellular carcinoma (HCC) (2). HBV encodes a partially double-stranded, circular DNA genome that is enclosed in an enveloped capsid. The HBV genome contains four overlapping open reading frames that encode the viral envelope, capsid, polymerase/reverse transcriptase, and nonstructural X (HBx) proteins. The results of studies from numerous groups suggest that the development of HBV-associated HCC likely involves both the consequences of immune-mediated destruction of HBV-infected hepatocytes and activities of HBV proteins, such as HBx (reviewed in references 21 and 73).

HBx is a multifunctional protein that can regulate HBV replication, cellular transcription, signal transduction pathways, proteasome activity, cell cycle progression, and apoptosis (35; reviewed in reference 11); however, whether these HBx activities contribute to the development of HBV-associated HCC is unresolved. Much of the confusion concerning the functions of HBx and its roles in HBV replication and HBV-associated HCC are likely a consequence of the numerous cell types and experimental conditions that have been used to analyze HBx activities; conclusions drawn from these various experimental systems have led to reports of discrepant HBx

activities. However, because the observed effects of HBx expression are probably influenced by cell-specific factors, it is likely that fundamental HBx activities are similar in different cell types, while the ultimate consequences of these activities are impacted by the characteristics of individual cells. This contention is strongly supported by reports from the laboratory of Andrisani and coworkers that demonstrate that HBx differentially affects cell signaling pathways depending on the extent of hepatocyte differentiation (44, 85, 86, 90).

The results of many studies have linked HBx expression to the activation of apoptotic pathways (20, 46, 57, 77, 80, 81, 83, 88, 90). For example, when human hepatoma Huh7 cells were transfected with an HBx expression vector, there was an increase in terminal transferase dUTP end labeling-positive nuclei and cytochrome *c* release from mitochondria, both markers of apoptosis (83). Studies in Chang cells, a human hepatocyte cell line, HepG2 cells, a human hepatoblastoma cell line, NIH 3T3 mouse fibroblasts, and MMDH3 cells, a mouse hepatocyte cell line, demonstrated that transiently expressed HBx sensitized these cells to various proapoptotic stimuli, such as tumor necrosis factor alpha (TNF- α), serum deprivation, oxidative stress, doxorubicine, or anti-Fas antibodies (38, 80, 81, 88). In NIH 3T3 cells, elevated levels of myc expression or ablation of NF- κ B signaling dramatically increased HBx activation of apoptotic pathways (81). Additional studies demonstrated that constitutively expressed HBx increased apoptosis in staurosporine- or cycloheximide (CHX)-treated Chang cells (4), while HBx that was expressed from a doxycycline-inducible promoter induced apoptosis in Chang cells when the activity of NF- κ B was inhibited (95).

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Directly opposing the proapoptotic activity of HBx that was observed in the studies described above is evidence from other studies that support a role for HBx in the inhibition of apoptosis (27, 30, 33, 47, 54, 63, 75). In one report, transiently transfected HBx inhibited apoptosis in serum-deprived or staurosporine- or etoposide-treated Chang cells (47). A similar study in Hep3B cells stably transfected with HBx showed that HBx also inhibited transforming growth factor β -induced apoptosis in these cells (75). HBx expression inhibited Fas-mediated apoptosis in DP-16 cells, a mouse erythroleukemia cell line, in mouse embryonic fibroblasts, and in Chang cells that constitutively expressed HBx (27). HBx also inhibited Fas-mediated apoptosis in HepG2 cells and in rat fibroblast REV2 cells and inhibited apoptosis in REV2 cells that were serum deprived or treated with TNF- α (33, 63).

Analyses of the effect of HBx expression on apoptotic pathways in HBx transgenic mouse models have also generated differing results; these differences likely reflect variations in the transgenic mouse strains, the promoters used to drive liver-specific HBx expression, and the levels of HBx expression at the time of analysis (40, 51, 87, 88). In HBx transgenic mice in which HBx was expressed under the control of the human α -1-antitrypsin regulatory region, no increase in hepatocyte apoptosis was observed (51). Conversely, hepatocytes of transgenic mice with HBx expressed under the control of the human antithrombin III promoter had a moderate increase in apoptosis (88); additional experiments in which these HBx transgenic mice were crossed with transgenic mice that overexpress Bcl-2 demonstrated that HBx inhibited Bcl-2-mediated protection from Fas activation of apoptosis (87). Apoptosis was also stimulated in transgenic mice expressing HBx under the control of its endogenous regulatory region (40).

Regulation of apoptosis involves the activation or inhibition of complex signaling cascades that are stimulated by factors that are intrinsic or extrinsic to the affected cell. One potential mechanism for HBx modulation of apoptotic pathways is through regulation of NF- κ B, a transcription factor that can stimulate the expression of antiapoptotic proteins (16, 17, 34, 41, 43, 58, 84). HBx activates NF- κ B in numerous cell types including hepatocytes, and studies have linked HBx activation of NF- κ B to HBx inhibition of TNF- α - and Fas-mediated stimulation of apoptotic pathways in NIH 3T3 cells, HepG2 cells, and Chang cells (19, 23, 79, 81, 92, 95). Apoptosis can also be regulated by modulation of the mitochondrial permeability transition pore (MPTP) (62). The MPTP is composed of several proteins including the voltage-dependent anion channel (VDAC), which spans the outer mitochondrial membrane, the adenine nucleotide translocator (ANT), which spans the inner mitochondrial membrane, and cyclophilin D, which is located on the inner surface of the inner mitochondrial membrane (62). The results of various studies have suggested that HBx and VDAC can interact (67, 68), and HBx-dependent regulation of the MPTP influences cytosolic calcium levels (55) and mitochondrial membrane depolarization (23). Modulation of the MPTP by HBx might also influence apoptotic pathways, although this could be either proapoptotic or antiapoptotic.

The results of the various studies described above highlight the potential impact of HBx on apoptotic pathways; however, the proapoptotic or antiapoptotic effects that have been observed in different cell types also emphasize the need for an

examination of the effect of HBx on apoptosis in authentic hepatocytes. Because transformed or immortalized cells often have altered signal transduction pathways, the ultimate consequence of a complex signaling cascade is likely to be different in these cells compared to normal hepatocytes. In addition, few studies have confirmed HBx apoptotic activities in the context of HBV replication.

In the work described here, we analyzed HBx modulation of apoptotic pathways in cultured primary rat hepatocytes, a biologically relevant system that closely mimics normal hepatocytes in the liver and is frequently used as a model for understanding human hepatocyte physiology (7, 12, 22, 25, 29, 36, 69). This system avoids cell line variations associated with transformation and immortalization and circumvents some of the difficulties in interpreting the results from apoptosis studies in available HBx transgenic mouse models where promoter variation can impact HBx expression levels at the time of analysis (40, 45, 87, 88). Because recent studies suggest that HBx continues to be expressed in the absence of other viral proteins or HBV replication in some HBV-associated liver tumors (64, 93), we studied the impacts of HBx on apoptotic pathways both when HBx was expressed in the absence of other HBV proteins and in the context of HBV replication; each of these conditions may be pertinent to the impact of HBx expression on hepatocyte physiology in various disease settings. Our studies demonstrate that in cultured primary rat hepatocytes, HBx inhibits apoptosis by activating NF- κ B but stimulates apoptotic pathways when NF- κ B activity is inhibited. HBx-induced activation of apoptotic pathways is through modulation of the MPTP. Similar results were observed when HBx was expressed in the context of HBV replication or in the absence of other HBV proteins. Our studies provide a link between the proapoptotic activity of HBx and its recently reported role in regulating the MPTP (23) and may explain the discrepant pro- and antiapoptotic activities of HBx that have been reported. Finally, the results of our studies in primary hepatocytes suggest a mechanistic link between HBV infections, HBx expression, and the development of liver cancer.

MATERIALS AND METHODS

Isolation and maintenance of primary rat hepatocytes in culture. Hepatocytes were isolated from male Sprague-Dawley rats as previously described (74). Cells were maintained at 37°C in 5% CO₂. Cultured rat hepatocytes were monitored for retention of hepatocyte morphology throughout the time course of our studies and were also collected for reverse transcriptase PCR (RT-PCR) analysis of hepatocyte-specific mRNAs (see below). Animal surgery and hepatocyte isolation complied with all relevant federal and institutional policies.

Transfections and reagents. Primary rat hepatocytes were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. A transfection efficiency of approximately 30 to 40% was routinely achieved; cotransfection of a green fluorescence protein (GFP) plasmid was included, where appropriate, to monitor transfection efficiency. All transfections were performed 24 h after plating. Where applicable, cells were transduced with equal infectious units of AdHBV or AdHBV(HBx-) (described below) 1 h posttransfection; examination of GFP-expressing cells at the time of cell collection confirmed equal infection efficiencies. Transfection followed by adenovirus infection has been shown to dramatically increase transfection efficiency through an adenovirus-mediated plasmid uptake (94). TNF- α was purchased from Calbiochem, and CHX, cyclosporine (CSA), and FK506 (Tacrolimus) were purchased from Sigma. Annexin V and 7-amino-actinomycin D (7-AAD) were purchased from Guava Technologies.

Antibodies. Anti-cytochrome *c* (A-8) antibody was purchased from Santa Cruz Biotechnology. Anti-Flag M2 antibody was purchased from Stratagene, and anti- β -actin antibody was purchased from Sigma. Anti-cleaved caspase 3 anti-

body was purchased from Cell Signaling, and anticore antibody was purchased from DakoCytomation, Inc.

Plasmids. FL1-154 HBx, which has N-terminally Flag-tagged (IBI, Inc.) full-length HBx cloned into the pcDNA3.1(–) vector, has been previously described (23). The IκB-α superrepressor (IκB-SR)-encoding plasmid has been previously described; this construct produces a mutant form of the IκB-α protein that has the serines at amino acid positions 32 and 36 mutated to alanines so that the mutant IκB cannot be phosphorylated and degraded (13, 15, 28, 89).

Generation of recombinant adenoviruses. Direct infection of rat hepatocytes with human HBV is not possible. However, adenoviruses can readily infect hepatocytes, and recombinant adenoviruses that contain a gene of interest can be used to transduce and express the product(s) of that gene in cultured primary hepatocytes. To facilitate expression of HBV and HBx in cultured hepatocytes, we generated recombinant adenoviruses that can transduce GFP (AdGFP), HBx (AdHBx), HBV (AdHBV), or an HBx-deficient HBV mutant [AdHBV(HBx–)]. The recombinant adenoviruses were constructed using the AdEasy System (Stratagene). pHRGFP (Stratagene) was first digested with NstI, blunted, digested with BglII, and then ligated into EcoRV- and BglII-digested pShuttle to generate pShuttleGFP. Consequently, all of the recombinant adenoviruses express GFP under the control of the major immediate-early cytomegalovirus promoter, allowing us to monitor the infection efficiency. HBx, the HBV 1.3-mer DNA sequence, or the HBV(HBx–) 1.3-mer DNA sequence were then cloned into XhoI-digested and blunted pShuttle-GFP. The protein-coding DNA sequence of HBx was isolated from pGEMHBV (71) and cloned into pShuttle-GFP; HBx expression is controlled by the simian virus 40 early promoter. The HBV 1.3-mer was derived from ApaI- and HindIII-digested pGEMHBV (payw1.2 [72]) or the HBx-deficient pGEMHBV(HBx–) (payw*7 [56]); therefore, all HBV RNAs are expressed under the control of endogenous promoters. The recombinant viruses lack the adenoviral E1 and E3 genes and are amplified in *trans*-complementing human embryonic kidney HEK293 cells. Recombinant adenoviruses were purified using Vivapure adenopack 20 (Sartorius Stedim Biotech) according to the manufacturer's directions, and viral titers were calculated according to the directions provided with the AdEasy vector system.

RT-PCR analysis of hepatocyte mRNAs. The authenticity of the hepatocytes was confirmed by detection of albumin (ALB), transferrin, hepatocyte nuclear factor 4 (HNF4), and connexin 26 (CNX 26) mRNAs, indicators of differentiated hepatocytes (8, 70). RNA was isolated from hepatocytes using TRIzol (Invitrogen) according to the manufacturer's instructions. The iScript cDNA synthesis kit (Bio-Rad) was used for the reverse transcription step as directed by the manufacturer. The primers used for the RT-PCRs and amplification protocols to detect HNF4, ALB, and transferrin mRNAs were previously described (23). We also included detection of CNX 26 as an additional marker of differentiated hepatocytes. The primers used for the connexin PCR were as follows: the forward oligonucleotide sequence for CNX 26 was 5'-GCAAGCTTGATTGGGGCACTAC-3', and the reverse oligonucleotide sequence was 5'-CGGATCCnGGACTTCCTGAGCAATACC-3'. RT-PCR analysis was conducted on hepatocytes immediately after isolation from perfused rat livers and on cultured hepatocytes 48 h posttransfection.

Annexin V and 7-AAD staining and fluorescence-activated cell sorting (FACS) analysis. Forty-eight hours posttransfection, living and dead hepatocytes were scraped into the cell growth medium and pelleted. For studies involving TNF-α, CHX, or CSA, these reagents were added 18 h prior to cell collection. Cells from one well of a six-well plate (~2 × 10⁵ cells) were resuspended in 600 μl of 1× Nexin buffer (Guava Technologies), and 50 μl of the suspended cells was then transferred to a new tube. Five microliters of annexin V stain and 5 μl of 7-AAD stain (Guava Technologies) were added to each tube and incubated for 20 min at room temperature in the dark. An additional 250 μl of 1× Nexin buffer was then added to each tube, and cells were analyzed on a Guava Technologies flow cytometer using the Nexin program according to the manufacturer's instructions.

Caspase 3 cleavage. Forty-eight hours following transfection, living and dead cells were scraped into the cell growth medium. For studies involving TNF-α or CHX, these reagents were added 18 h prior to cell collection. Cells were pelleted at 2,000 rpm for 4 min at 4°C and then lysed in 2% sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 240 mM Tris [pH 6.8], and 10% glycerol). The entire sample was loaded on a 15% polyacrylamide gel, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred to a nitrocellulose membrane (Bio-Rad), followed by blocking for 2 h in 5% milk and incubation with primary antibody overnight. The membrane was then washed and incubated with a horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence (ECL) system (Bio-Rad). Western blot analysis was conducted with antibodies specific for cleaved caspase 3, β-actin, or Flag. Blots were quantified using EZ-Quant software (EZQuant, Ltd.), and differences in expression were calculated by dividing

the quantification results from the HBx-expressing cells by the results from the control cells. Statistical analysis, conducted using Student's *t* test, verified whether differences were statistically significant ($P \leq 0.05$).

Cytochrome *c* release. Forty-eight hours posttransfection, living and dead cells were scraped into the culture medium. For studies involving TNF-α, CHX, or CSA, these reagents were added 18 h prior to cell collection. Cells were pelleted at 2,000 rpm for 4 min at 4°C followed by isolation of a cytosolic fraction using the mitochondrial isolation kit for mammalian cells (Pierce) according to the manufacturer's instructions. Briefly, following lysis of approximately 1 × 10⁶ primary hepatocytes, cell debris/nuclei were pelleted and discarded, and then the supernatant was separated into mitochondrial and cytosolic fractions by centrifugation at 10,000 × *g*. The pellet from the 10,000 × *g* centrifugation is the mitochondrial fraction, and the supernatant is the cytosolic fraction. Approximately one-eighth of the cytosolic fraction was separated by SDS-PAGE on a 15% polyacrylamide gel. Western blot analysis was conducted using β-actin as a loading control, a cytochrome *c* (A-8) antibody to detect cytochrome *c*, and an anti-Flag antibody to detect FL1-154 HBx. Blots were quantified using EZ-Quant software (EZQuant, Ltd.), and statistical analysis, conducted using Student's *t* test, verified whether differences were statistically significant ($P \leq 0.05$).

HBV core protein Western blot analysis. Hepatocytes were collected 48 h posttransfection and lysed in 2% SDS lysis buffer. Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad), followed by blocking for 2 h in 5% milk and incubation with antibodies specific for β-actin or HBV core protein.

Luciferase assay. Twenty-four hours after plating, primary hepatocytes were transfected with IκB-α-SR, pNF-κB-Luc, or pNF-κB-Luc-MUT followed by infection with AdHBV or AdHBV(HBx–), where appropriate. The NF-κB-Luc reporter vectors were a gift from E. Skolnik. Luciferase reporter assays were performed 48 h posttransfection using the Promega luciferase assay system according to the manufacturer's instructions.

Replication assay. Twenty-four hours after plating, cultured primary rat hepatocytes were transfected with FL1-154 HBx or pcDNA3.1(–) followed by infection with AdHBV or AdHBV(HBx–) 3 h posttransfection. Alternately, 24 h after plating, cells were infected with AdHBV, AdHBV(HBx–), AdHBx, or AdGFP, as appropriate. Forty-eight hours postinfection, cells were collected, and HBV replication was analyzed by Southern blotting as previously described (9).

Northern blot analysis. Twenty-four hours after plating, cultured primary rat hepatocytes were infected with AdHBV or AdHBV(HBx–). Cells were collected 48 h postinfection, and total RNA was isolated from hepatocytes using TRIzol (Invitrogen), according to the manufacturer's instructions. Poly(A)⁺ RNA was isolated using oligo(dT)-cellulose columns (Molecular Research Center, Inc.), according to the manufacturer's instructions, followed by Northern blot analysis as previously described (10).

RESULTS

Confirmation of hepatocyte differentiation in culture. To confirm that the cultured primary rat hepatocytes remained differentiated during the course of this study, cell morphology was continually monitored, and RT-PCR analysis was performed to detect expression of the hepatocyte-specific markers, transferrin, albumin, hepatocyte nuclear factor 4, and connexin 26 (8, 65, 70). Hepatocyte morphology was maintained throughout the experiment, and expression of each hepatocyte-specific marker was observed in freshly isolated hepatocytes and in cultured hepatocytes 48 h posttransfection, the time of cell collection (Fig. 1). These results are consistent with other published studies that have used similar isolation and culture techniques to maintain differentiated rat hepatocytes (7, 22, 23, 29, 36, 69).

HBx inhibits apoptosis in primary rat hepatocytes. Multiple assays of apoptosis were conducted to examine the effect of HBx on apoptosis. Initially, primary rat hepatocytes were transfected with FL1-154 HBx or pcDNA3.1(–), and 48 h posttransfection, cells were stained with annexin V and 7-AAD, markers of early and late-stage apoptosis, respectively. There is a low level of spontaneous apoptosis in cultured primary rat hepatocytes (12), and the results of FACS analysis showed that HBx

FIG. 1. Confirmation of hepatocyte differentiation in culture. RNA was extracted from primary rat hepatocytes either freshly isolated from a rat liver or from cultured primary rat hepatocytes maintained as described in Materials and Methods. RT-PCR analysis was performed to analyze expression of albumin (ALB), transferrin (TFN), hepatocyte nuclear factor 4 (HNF4), and connexin 26 (CNX 26) as markers of differentiated hepatocytes. Expected band sizes are 914 bp for ALB, 530 bp for TFN, 770 bp for HNF4, and 663 bp for CNX 26. Note that the primers for ALB and TFN were combined in the same sample due to the substantial difference in PCR product size. Lanes with (–) at the beginning of the lane label contain samples in which isolated RNA was analyzed directly by PCR to confirm the absence of contaminating DNA.

inhibited this spontaneous apoptosis (Fig. 2A). We next examined whether HBx could also inhibit TNF- α -induced apoptosis; this cytokine is expressed at high levels in the liver during a chronic HBV infection (32). Although apoptosis is dramatically increased in primary hepatocytes by the addition of TNF- α and CHX, a protein synthesis inhibitor, TNF- α alone

can also be used to induce a low level of apoptosis in cultured primary rat hepatocytes (12, 76). TNF- α treatment increased apoptosis in control transfected hepatocytes compared to untreated hepatocytes; however, in HBx-expressing hepatocytes, TNF- α -induced apoptosis was inhibited (Fig. 2A). Although the HBx-induced changes in spontaneous and TNF- α -induced apoptosis levels were small, they were consistently observed and statistically significant ($P \leq 0.05$).

To provide additional evidence that HBx expression inhibited apoptosis in cultured primary hepatocytes, we monitored the level of cleaved caspase 3. Cleaved caspase 3 is generated as part of the cellular proapoptotic cascade and is considered an indicator of late-stage apoptosis (61). HBx-expressing or control hepatocytes were collected 48 h posttransfection, and whole-cell lysates were separated by SDS-PAGE followed by Western blot analysis with an antibody to cleaved caspase 3. The levels of cleaved caspase 3 that resulted from spontaneous apoptosis were lower in HBx-expressing primary rat hepatocytes than in control transfected hepatocytes (Fig. 2B). The effect of HBx on TNF- α -induced activation of apoptosis and the resultant increase in the levels of cleaved caspase 3 were also examined. TNF- α treatment increased caspase 3 cleavage in control transfected hepatocytes compared to untreated cells; however, HBx inhibited the TNF- α -induced increase in the levels of cleaved caspase 3 (Fig. 2B).

For a final marker for activation of apoptotic pathways, we monitored release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. To examine cytochrome *c* release, cytosolic and mitochondrial fractions were isolated from HBx-expressing or control primary hepatocytes. The frac-

FIG. 2. HBx inhibits spontaneous and TNF- α -induced apoptosis in primary rat hepatocytes. Primary rat hepatocytes were transfected with FL1-154 HBx (HBx) or pcDNA3.1 [3.1(-)]. Hepatocytes were treated with TNF- α , where applicable, 30 h posttransfection. Hepatocytes were collected 48 h posttransfection. (A) Cells were stained with annexin V and 7-AAD, followed by FACS analysis. Statistical analysis, conducted using Student's *t* test, verified that these differences are statistically significant ($P \leq 0.05$). Error bars represent the standard errors. (B) Whole-cell lysate was resolved by SDS-PAGE and subjected to Western blot analysis with anti- β -actin, anti-cleaved caspase 3, or anti-Flag antibodies. Cl Casp 3, cleaved caspase 3. (C) Cytosolic fractions were isolated from HBx-expressing or control hepatocytes. Fractions were resolved by SDS-PAGE, followed by Western blot analysis for β -actin, cytochrome *c* (cyt *c*), or Flag. Differences in expression (*n*-fold) were calculated as described in Materials and Methods and are shown below the gels in panels B and C. Statistical analysis was conducted using Student's *t* test. *, statistically significant difference (*n*-fold) between results for control and HBx-expressing cells ($P \leq 0.05$).

FIG. 3. HBx induces apoptosis in the absence of NF- κ B activity. (A) HBx-expressing or control cells were treated with cycloheximide (CHX) 18 h prior to cell collection; cells were collected 48 h posttransfection. The whole-cell lysates were resolved by SDS-PAGE, followed by Western blot analysis for β -actin, cleaved caspase 3 (Cl casp 3), or HBx-Flag. (B) Primary rat hepatocytes were cotransfected with FL1-154 HBx (HBx) or pcDNA3.1(-) [3.1(-)] and I κ B-SR (SR). Cells were collected 48 h posttransfection and stained with annexin V or 7-AAD, followed by FACS analysis. Statistical analysis was conducted using Student's *t* test. *, statistically significant difference (*n*-fold) between results for control and HBx-expressing cells ($P \leq 0.05$). (C) Hepatocytes were cotransfected with FL1-154 HBx or pcDNA3.1(-) and I κ B-SR (+SR). Cells were collected 48 h posttransfection, and whole-cell lysates were resolved by SDS-PAGE, followed by Western blot analysis for β -actin, cleaved caspase 3, or HBx. (D) Hepatocytes were cotransfected with FL1-154 HBx or pcDNA3.1(-) and I κ B-SR. Cells were collected 48 hours posttransfection, and a cytosolic fraction was isolated and separated by SDS-PAGE. Western blot analysis was conducted with anti- β -actin, anti-cytochrome *c*, or anti-Flag antibodies. Differences in expression (*n*-fold) were calculated as described in Materials and Methods and are shown below the gels.

tions were resolved by SDS-PAGE, and Western blot analysis was performed using a cytochrome *c*-specific antibody. Less cytosolic cytochrome *c* was observed in HBx-transfected hepatocytes than in control hepatocytes in assays of spontaneous apoptosis (Fig. 2C). The antiapoptotic effect of HBx was even more apparent in the presence of TNF- α treatment (Fig. 2C). Because we consistently observed a direct correlation between the increase in cytosolic cytochrome *c* and the decrease in mitochondrial cytochrome *c* (data not shown), we analyzed cytochrome *c* expression only in the cytosolic fraction for all subsequent studies. Cumulatively, the results of the various assays of apoptosis that were conducted demonstrate that HBx can inhibit both spontaneous and TNF- α -induced apoptosis in cultured primary rat hepatocytes.

HBx induces apoptosis in the absence of protein synthesis or NF- κ B activity. We previously reported that HBx activated NF- κ B and prevented mitochondrial membrane depolarization in primary rat hepatocytes, whereas HBx induced mitochondrial membrane depolarization when HBx-expressing primary rat hepatocytes were either exposed to CHX or transfected with a vector encoding a mutant I κ B- α (I κ B-SR) that inhibits NF- κ B signaling (23). Because mitochondrial membrane potential regulation and the activity of NF- κ B have been associated with modulation of apoptotic pathways, we next asked whether HBx lost its antiapoptotic activity when HBx-expressing hepatocytes were exposed to CHX or transfected with an I κ B-SR expression vector.

As an initial simple assessment of apoptotic activity, we first assayed the levels of cleaved caspase 3 in HBx-expressing

hepatocytes that were exposed to CHX treatment. The collected hepatocytes were lysed, and cleaved caspase 3 in the whole-cell lysates was analyzed by SDS-PAGE and Western blot analysis. In the presence of CHX, HBx no longer inhibited caspase 3 cleavage; rather, HBx induced cleavage of caspase 3 (Fig. 3A). In agreement with our previously published data, the levels of HBx were not different in CHX-treated or control cells (23). These results suggest that in the absence of protein synthesis, HBx loses its antiapoptotic activity and becomes proapoptotic; exposure to TNF- α was not required for this proapoptotic activity. These results also correlate with the previously reported alteration in HBx regulation of mitochondrial membrane potential that was observed in CHX-treated, HBx-expressing primary rat hepatocytes (23). Because CHX treatment inhibits NF- κ B-induced synthesis of antiapoptotic proteins and because HBx was previously reported to activate NF- κ B in primary rat hepatocytes (23), these results also suggest that the antiapoptotic activity of HBx may be linked to its activation of NF- κ B.

To directly assess whether the antiapoptotic activity of HBx is associated with activation of NF- κ B, we next examined the apoptotic activity of HBx in cultured primary rat hepatocytes when NF- κ B signaling was inhibited. NF- κ B activity was inhibited by transfecting hepatocytes with a plasmid that expresses I κ B-SR. Hepatocytes were cotransfected with control vectors or FL1-154 HBx and I κ B-SR, and 48 h posttransfection, cells were analyzed by annexin V or 7-AAD staining and FACS analysis. In contrast to the antiapoptotic activity of HBx that was observed in control transfected cells, in the absence of

NF- κ B activity, HBx expression induced apoptosis (Fig. 3B). We also analyzed cleavage of caspase 3 and cytosolic levels of cytochrome *c*. When HBx was cotransfected with I κ B-SR, the levels of cleaved caspase 3 were increased in comparison to the levels in hepatocytes that were transfected with the control vector and I κ B-SR as well compared to hepatocytes that were not transfected with I κ B-SR (Fig. 3C). In agreement with our previously published observations, HBx expression was not impacted by cotransfection with I κ B-SR (Fig. 3) (23). Similar results were obtained when the levels of cytosolic cytochrome *c* were analyzed in HBx-expressing cells in which NF- κ B signaling was inhibited (Fig. 3D). Overall, these results demonstrate that HBx inhibits spontaneous and TNF- α -induced apoptosis in primary rat hepatocytes by activating NF- κ B, but that it induces apoptosis when NF- κ B activity is inhibited.

In the studies shown in Fig. 2 and 3, we noted that the results of Western blot analyses for apoptotic markers consistently showed more notable differences than observed in FACS studies; this could be due to a greater sensitivity of the Western blot analysis compared to the sensitivity of annexin V or 7-AAD staining and FACS analysis. In addition, the observable differences in the levels of annexin V and 7-AAD in HBx-expressing and control hepatocytes that were measured by FACS analysis could be diminished by high levels of autofluorescence in cultured primary hepatocytes (24; A. J. Clippinger and M. J. Bouchard, unpublished observations). While the results with the cleaved caspase 3 antibodies consistently agreed with cytochrome *c* release and annexin V or 7-AAD studies, we also noted considerable lot-to-lot variation in the sensitivity of the cleaved caspase 3 antibodies. Therefore, in subsequent HBx and HBV studies, we analyzed apoptosis with cytochrome *c* release assays and some annexin V or 7-AAD studies.

HBx regulates the MPTP to induce apoptosis in the absence of NF- κ B activity. Regulation of the mitochondrial permeability transition pore has been directly linked to modulation of apoptotic pathways. The results from several previous studies suggest a role for HBx in the regulation of the MPTP, potentially through its reported interaction with the voltage-dependent anion channel (9, 23, 55). We previously reported that the ability of HBx to induce mitochondrial membrane depolarization in the absence of NF- κ B signaling was associated with an HBx-dependent modulation of the MPTP. These studies provided a mechanistic link between HBx modulation of the MPTP and its ability to modify mitochondrial membrane potential. We now asked whether HBx regulation of the MPTP might also be linked to its proapoptotic activity in primary rat hepatocytes. HBx-expressing or control hepatocytes were treated with CSA, which interacts with cyclophilin D, a component of the MPTP, and inhibits MPTP activities (14). Hepatocytes were cotransfected with various combinations of the control vector or FL1-154 HBx and I κ B-SR, and 30 h posttransfection, CSA was added to the appropriate samples. Cells were collected 18 h later (48 h posttransfection) for FACS analysis of annexin V or 7-AAD staining. In the presence of CSA, HBx did not induce apoptosis in cells cotransfected with HBx and I κ B-SR, demonstrating that the proapoptotic activity of HBx is regulated by an MPTP-dependent mechanism (Fig. 4A). Finally, because CSA inhibits both cyclophilin D and calcineurin, FK506, a calcineurin inhibitor that does not affect

the MPTP, was also used to show that CSA inhibition of the MPTP was directly responsible for blocking the proapoptotic activity of HBx (26). Unlike CSA treatment, FK506 treatment of hepatocytes cotransfected with HBx and I κ B-SR did not abrogate the HBx-induced activation of apoptosis (Fig. 4A). Additionally, neither CSA treatment nor FK506 treatment altered the HBx-dependent inhibition of apoptosis when NF- κ B activity was not inhibited (Fig. 4A). For an additional indicator of stimulation of proapoptotic pathways, we also examined the levels of cytosolic cytochrome *c* in control hepatocytes and hepatocytes cotransfected with HBx and I κ B-SR with or without CSA treatment. CSA treatment abrogated HBx-induced cytochrome *c* release from mitochondria in the absence of NF- κ B activity (Fig. 4B), confirming that regulation of the MPTP plays a role in HBx-dependent activation of apoptotic pathways. Additionally, FK506 treatment in the presence of I κ B-SR did not affect HBx-induced cytochrome *c* release, and neither CSA treatment nor FK506 treatment altered the HBx-dependent inhibition of apoptosis when NF- κ B activity was not blocked (Fig. 4B). These results suggest that HBx can either induce or inhibit apoptosis, depending on the status of NF- κ B in hepatocytes. In cultured primary rat hepatocytes, HBx-dependent activation of NF- κ B inhibits apoptosis; however, when NF- κ B activity is inhibited, HBx induces apoptosis through an MPTP-dependent mechanism.

HBV replication in cultured primary hepatocytes is regulated by HBx expression. To study the impact of HBV replication and associated HBx expression on hepatocyte apoptosis, we first constructed an efficient HBV replication system. Transfection of hepatocytes with the 1.3-mer cDNA that is often used for studies in HepG2 cells resulted in very low levels of HBV replication that were often difficult to detect (M. J. Bouchard, unpublished observations). To circumvent this and develop a robust replication system, we generated recombinant adenoviruses that contain the 1.3-mer HBV DNA (AdHBV) and the 1.3-mer HBV that lacks expression of HBx [AdHBV(HBx-)]. Cells were transduced with equal levels of AdHBV or AdHBV (HBx-) so that 100% of the cultured hepatocytes were infected; all the adenoviruses also expressed GFP so that the efficiency of adenoviral infection of cultured primary hepatocytes could be monitored (see Materials and Methods). To minimize any potential adenovirus effects, the minimum amount of virus required to infect 100% of cells was used. HEK293 cells express the adenovirus E1A region, are used to expand stocks of recombinant adenovirus, and are used for determining the titers of the recombinant adenovirus stocks. Because the recombinant adenoviruses do not replicate in cultured primary rat hepatocytes, it was not possible to determine the viral titers in rat hepatocytes. Therefore, we used titers that were determined with HEK293 cells as a reference to ensure that equivalent numbers of virus were added from each recombinant adenovirus stock and then tested various amounts of our stock of purified viruses to determine the lowest amount required to infect 100% of the hepatocytes. We noted that cultured primary rat hepatocytes were more susceptible to infections with the recombinant adenoviruses than HEK293 cells were, and 10-fold-lower levels of purified virus preparations were required to infect 100% of a sample of cultured primary rat hepatocytes than were required to infect an equal number of HEK293 cells.

FIG. 4. HBx induces apoptosis in the absence of NF- κ B activity through an MPTP-dependent mechanism. Primary rat hepatocytes were transfected with FL1-154 HBx (HBx), pcDNA3.1(-) [3.1(-)], and/or I κ B-SR (SR). Hepatocytes were treated with CSA or FK506, where applicable, 30 h posttransfection. Hepatocytes were collected 48 h posttransfection. (A) Cells were stained with annexin V and 7-AAD, followed by FACS analysis. Statistical analysis, conducted using Student's *t* test, verified that these differences are statistically significant ($P \leq 0.05$) as indicated by an asterisk. Error bars represent the standard errors. Note that the SR bar graph from Fig. 3B is included in this figure for comparison purposes. (B) Cytosolic fractions were separated by SDS-PAGE, followed by Western blot analysis for β -actin, cytochrome *c* (cyt *c*), or HBx-Flag. Differences in expression (*n*-fold) were calculated as described in Materials and Methods and are shown below the gels. Statistical analysis was conducted using Student's *t* test. *, statistically significant difference (*n*-fold) between results for HBx-expressing and control cells ($P \leq 0.05$).

When hepatocytes were infected with AdHBV and collected 48 h later for analysis of HBV replication, robust HBV replication was observed. However, when cultured primary rat hepatocytes were infected with equal levels of AdHBV(HBx-) for the same time period, only very low levels of HBV replication were apparent (Fig. 5A). Replication of the AdHBV

(HBx-)-infected cells could be rescued by transfection of these hepatocytes with FL1-154 HBx (data not shown) or by coinfection with AdHBx (Fig. 5A). These results demonstrate that HBV replication in cultured primary rat hepatocytes is strongly influenced by the expression of HBx and that the recombinant adenovirus system can be used to study the phys-

FIG. 5. Replication of an HBx-deficient HBV is rescued by HBx expression in primary rat hepatocytes. (A) HBV replication was monitored by Southern blot analysis 48 h postinfection. HBx expression rescued replication from AdHBV(HBx-) to wild-type levels, while expression of the negative control did not. (B) Hepatocytes were infected with equal amounts of AdHBV and AdHBV(HBx-) or with an increased amount of AdHBV(HBx-). Viral RNAs were monitored by Northern blot analysis 48 h postinfection. (C) Hepatocytes were infected with equal amounts of AdHBV and AdHBV(HBx-) or with an increased amount of AdHBV(HBx-). Cells were collected 48 h postinfection, and Western blot analysis was conducted using an anticore antibody. (D) HBV replication was monitored by Southern blot analysis 48 h postinfection. Hepatocytes were infected with equal amounts of AdHBV and AdHBV(HBx-) or with an increased amount of AdHBV(HBx-).

iological impact of HBx expressed in the context of HBV replication. Northern and Western blot analyses were conducted to determine whether infection of primary rat hepatocytes with AdHBV and AdHBV(HBx-) produced similar amounts of viral RNAs and core protein (Fig. 5B and C). In agreement with a recent study of HBx-dependent HBV replication in mice (37), rat hepatocytes infected with AdHBV(HBx-) produced lower levels of viral RNAs and core protein than hepatocytes infected with the same amount of AdHBV viral particles did (Fig. 5B and C). When primary rat hepatocytes were infected with threefold-more AdHBV(HBx-) than AdHBV, we observed equal levels of viral RNAs and core protein (Fig. 5B and C) but still did not observe equivalent levels of HBV replication (Fig. 5D). It is important to note that HBV transcripts and proteins in this system are likely derived from the HBV DNA sequence that is integrated into the recombinant adenovirus; however, our results still demonstrate that HBx expression impacts HBV replication in primary hepatocytes by regulating both the levels of viral RNAs and a posttranscriptional replication process.

HBx expressed in the context of HBV replication inhibits hepatocyte apoptosis. We next examined whether HBx regulated apoptotic pathways when it was expressed in the context of HBV replication. HBx is expressed at very low levels from the HBV genome and, in our experience, is difficult to detect unless large numbers of HBV-infected primary rat hepatocytes are analyzed in a single Western blot analysis (23). This makes it impractical to conduct all the experiments outlined below and detect HBx in every experimental condition; however, our replication assays with AdHBV- and AdHBV(HBx-)-infected primary rat hepatocytes provide compelling evidence that HBx is expressed from AdHBV and that the only defect for HBV replication in AdHBV(HBx-)-infected cells is the absence of HBx expression. From our experience with the different apoptotic assays that were described above in studies of HBx-transfected primary rat hepatocytes, we determined that cytochrome *c* release from mitochondria was the most sensitive and consistent assay in this system; for this reason and because the results of studies described above demonstrate a direct correlation between the levels of cytosolic cytochrome *c*, cleaved caspase 3, and annexin V or 7-AAD staining, we assayed the levels of cytosolic cytochrome *c* as an indicator of activation of apoptotic pathways in AdHBV- and AdHBV(HBx-)-infected hepatocytes.

Cultured primary rat hepatocytes were infected with equal amounts of AdHBV or AdHBV(HBx-), and 48 h postinfection, the hepatocytes were collected, and a cytosolic fraction was isolated and analyzed by Western blot analysis to determine the levels of cytosolic cytochrome *c*. Similar to results in HBx-transfected primary rat hepatocytes, less spontaneous induction of apoptosis, as measured by cytosolic cytochrome *c*, was observed in AdHBV-infected hepatocytes than in AdHBV(HBx-)-infected hepatocytes, demonstrating that HBx also inhibits cytochrome *c* release when expressed in the context of HBV replication (Fig. 6A). The antiapoptotic effect of HBV was even more apparent in the presence of TNF- α treatment (Fig. 6A). For these and subsequent studies, we used equal levels of AdHBV and AdHBV(HBx-) for all apoptotic studies. However, to ensure that the absence of an antiapoptotic effect in AdHBV(HBx-)-infected cells was not due to lower expression of other viral proteins, we also tested increased levels of AdHBV(HBx-) that resulted in equivalent viral

FIG. 6. HBx inhibits spontaneous and TNF- α -induced cytochrome *c* release from mitochondria when expressed in the context of HBV replication. (A) Primary rat hepatocytes were infected with equal amounts of AdHBV (HBV) or AdHBV(HBx-) [HBV(HBx-)]. Where applicable, cells were treated with TNF- α for 18 h before cell collection. A cytosolic fraction was isolated from hepatocytes 48 h postinfection, resolved by SDS-PAGE, and subjected to Western blot analysis with anti- β -actin, anti-cytochrome *c*, or anti-Flag antibodies. cyt *c*, cytochrome *c*. (B) Primary rat hepatocytes were infected with equal amounts of AdHBV and AdHBV(HBx-) or with an increased level of AdHBV(HBx-) [$\uparrow\uparrow$ HBV(HBx-)], which results in the production of equivalent viral RNA and core protein levels. A cytosolic fraction was isolated 48 h postinfection, resolved by SDS-PAGE, and subjected to Western blot analysis with anti- β -actin or anti-cytochrome *c* antibodies. Differences in expression (*n*-fold) were calculated as described in Materials and Methods and are shown below the gels. Statistical analysis was conducted using Student's *t* test. *, statistically significant difference (*n*-fold) between results for control and HBx-expressing cells ($P \leq 0.05$).

RNA and core protein levels. This elevated level of AdHBV(HBx-) did not cause an antiapoptotic effect, demonstrating that the observed antiapoptotic effect in AdHBV-infected cells was caused by HBx expression (Fig. 6B).

HBV activates NF- κ B in primary rat hepatocytes. As a first step in determining whether HBV activates NF- κ B to modulate apoptosis, we used a NF- κ B-dependent luciferase reporter construct to assay HBV activation of NF- κ B in primary rat hepatocytes. Primary hepatocytes were cotransfected with either wild-type pNF- κ B-Luc or pNF- κ B-Luc-MUT and I κ B-SR, where applicable, followed by infection with AdHBV or AdHBV(HBx-). Cells were collected 48 h posttransfection and processed using the Promega luciferase assay system, and luciferase activity was measured using a luminometer. A 2.5-fold induction in NF- κ B activation was observed in HBV-expressing hepatocytes compared to HBV(HBx-)-expressing hepatocytes (Fig. 7). This activation was not observed using plasmid pNF- κ B-Luc-MUT or with I κ B-SR, regardless of HBV expression (Fig. 7). These results are similar to our previously reported results showing HBx-induced activation of NF- κ B in primary rat hepatocytes (23).

HBx expressed in the context of HBV replication stimulates apoptosis in the absence of NF- κ B activity. We next determined whether the antiapoptotic activity of HBV is associated

FIG. 7. HBV induces NF-κB activation in primary rat hepatocytes. Twenty-four hours after plating, primary rat hepatocytes were transfected (+) with pNF-κB-Luc (NF-κB-Luc) (wild type [wt]), pNF-κB-Luc-MUT (NF-κB-Luc-MUT) (mutant [mut]), or IκB-SR, where applicable, followed by infection (+) with AdHBV (HBV) or AdHBV(HBx-) [HBV (HBx-)]. Cells were collected 48 h posttransfection, and luciferase assays were performed as described in Materials and Methods. This is a representative graph from three separate experiments, each performed in triplicate. Error bars represent the standard errors.

with HBx-dependent NF-κB activation. We examined the effect of HBx on apoptosis when NF-κB was inhibited by transfecting hepatocytes with a plasmid that expresses IκB-SR. Hepatocytes were transfected with IκB-SR and then trans-

duced with equal amounts of AdHBV or AdHBV(HBx-) 1 h posttransfection. Forty-eight hours posttransfection, cells were collected and analyzed for cytochrome *c* release by Western blot analysis. In contrast to the antiapoptotic activity observed in HBV-expressing hepatocytes, in the absence of NF-κB activity, HBV expression induced cytochrome *c* release (Fig. 8A). We also examined apoptosis following infection with an increased amount of AdHBV(HBx-) that produces levels of viral RNAs and core protein similar to those produced by AdHBV-infected hepatocytes (Fig. 5B and C). Our results demonstrate that there is more cytochrome *c* released in the HBV-expressing, IκB-SR-transfected cells than in the IκB-SR-transfected cells infected with an increased amount of HBV(HBx-) (Fig. 8B); these results are similar to our results using lower levels of AdHBV(HBx-) (Fig. 8A). Overall, these results show that HBx expressed in the context of HBV replication stimulates cytochrome *c* release in the absence of NF-κB activity.

HBx expressed in the context of HBV replication regulates the MPTP to induce apoptosis in the absence of NF-κB activity. We next examined whether HBx expressed in the context of replication regulates the MPTP to induce apoptosis. Hepatocytes were transfected with IκB-SR and then transduced with equal amounts of AdHBV or AdHBV(HBx-) 1 h posttransfection followed by treatment with CSA 18 h prior to cell collection. Forty-eight hours posttransfection, cells were collected and analyzed for cytochrome *c* release by Western blot

FIG. 8. HBx expressed in the context of HBV replication induces cytochrome *c* (cyt *c*) release in the absence of NF-κB activity. (A) Primary rat hepatocytes were infected with equal amounts of AdHBV (HBV) or AdHBV(HBx-) [HBV(HBx-)]. Where applicable, hepatocytes were first transfected with IκB-SR (+SR), followed by infection with AdHBV or AdHBV(HBx-) 1 hour later. Hepatocytes were treated with CSA or FK506, where applicable, 30 h postinfection. A cytosolic fraction was isolated from hepatocytes 48 h postinfection, resolved by SDS-PAGE, and subjected to Western blot analysis with anti-β-actin or anti-cytochrome *c* antibodies. (B) Primary rat hepatocytes were transfected with IκB-SR, followed by infection 1 hour later with equal amounts of AdHBV or AdHBV(HBx-) or with an increased amount of AdHBV(HBx-) [↑↑ HBV(HBx-)] that produces levels of viral RNAs and core protein equal to those produced by AdHBV. A cytosolic fraction was isolated 48 h postinfection, resolved by SDS-PAGE, and subjected to Western blot analysis with anti-β-actin or anti-cytochrome *c* antibodies. Differences in expression (*n*-fold) differences were calculated as described in Materials and Methods and are shown below the gels. Statistical analysis was conducted using Student's *t* test. *, statistically significant difference (*n*-fold) between results for control and HBx-expressing cells (*P* ≤ 0.05).

analysis. CSA abrogated the induction of cytochrome *c* release in AdHBV-infected and I κ B-SR-transfected hepatocytes (Fig. 8A). A FK506 control was included to ensure that the CSA treatment results were due to its inhibition of cyclophilin D and not of calcineurin (Fig. 8A). Additionally, CSA or FK506 treatment in the absence of I κ B-SR did not affect the HBx-dependent inhibition of cytochrome *c* release (Fig. 8A). Taken together, these results suggest that HBx, when expressed on its own and in the context of HBV replication, inhibits apoptosis in primary rat hepatocytes through the activation of NF- κ B; however, in the absence of NF- κ B activity, HBx expression induces apoptosis through an MPTP-dependent process.

DISCUSSION

Chronic HBV infections are associated with the development of hepatocellular carcinoma (2). The smallest HBV open reading frame encodes the nonstructural X protein HBx; HBx is a multifunctional HBV protein that regulates HBV replication and numerous cellular signal transduction pathways (11). HBx regulation of HBV replication and cell signaling pathways involves both direct interactions with cellular factors as well as indirect activation of signaling pathways, often through modulation of cytosolic calcium levels (11). While still the subject of debate, HBx is thought to contribute to the development of HBV-associated HCC. Numerous reports have suggested that HBx can regulate apoptotic pathways, providing a potential mechanistic link between HBx expression and the development of HBV-associated HCC (20, 27, 30, 33, 46, 47, 54, 57, 63, 75, 77, 80, 81, 83, 88, 90). However, the reported effects of HBx on apoptosis have varied, depending on the experimental system in which these activities were analyzed, and whether HBx regulates apoptosis in authentic hepatocytes is not entirely understood.

We have characterized HBx regulation of apoptosis in cultured primary rat hepatocytes, a biologically relevant system for studying hepatocyte physiology and apoptosis (7, 12, 22, 25, 29, 36, 69). This model system circumvents potential problems associated with previously reported studies in HBx transgenic mice or in transformed or immortalized cell lines. We closely monitored the cultured primary rat hepatocytes throughout the time course of our studies to ensure that cell morphology and expression of hepatocyte-specific markers were consistent with the phenotype of differentiated hepatocytes (Fig. 1). In addition, because our studies were conducted in transiently transfected hepatocytes, adaptation of hepatocytes to continued expression of HBx, which might be present in cell lines that constitutively express HBx or in HBx transgenic mouse models, was unlikely to occur during the short time frame of our studies. Finally, we also confirmed that the observed consequences of HBx expression were apparent in the context of HBV replication, supporting the notion that the impact of HBx on hepatocyte apoptotic pathways was not an artifact of HBx overexpression in our system.

The effect of HBx on apoptosis in primary hepatocytes was monitored with annexin V and 7-AAD staining, cytochrome *c* release from mitochondria, and caspase 3 activation. Low levels of spontaneous apoptosis are observed in cultures of primary rat hepatocytes, and HBx inhibited this spontaneous apoptosis (Fig. 2). HBx also inhibited the moderate increase in

hepatocyte apoptosis that was observed after exposure to TNF- α , a cytokine that is produced at high levels during the immune response to an HBV infection (Fig. 2) (32). Similar results were observed when cells were infected with recombinant adenoviruses that transduced HBV (AdHBV) or with an HBV that lacked HBx expression [AdHBV(HBx-)] (Fig. 6). As a consequence of these studies, we also demonstrated HBx-dependent HBV replication in cultured primary rat hepatocytes (Fig. 5A). These results are consistent with *in vivo* and *in vitro* studies in other experimental systems and provide an additional model for studying the impact of HBx expression on HBV replication in normal hepatocytes (18, 56, 96). Overall, HBx expression strongly stimulated HBV replication in primary rat hepatocytes but inhibited spontaneous and TNF- α -induced apoptosis.

To begin to understand the mechanism associated with HBx modulation of apoptosis, we treated HBx-expressing primary rat hepatocytes with cycloheximide, an inhibitor of protein synthesis. In the presence of CHX, HBx no longer inhibited caspase 3 cleavage; rather, HBx induced caspase 3 cleavage (Fig. 3A). These results show that continual protein synthesis is required for HBx-dependent inhibition of apoptosis and that in the absence of new protein synthesis, HBx stimulates apoptosis. Because CHX treatment can inhibit the synthesis of NF- κ B-regulated proteins and because HBx activates NF- κ B in various established cell lines and in primary rat hepatocytes, we next examined the role of NF- κ B activity in HBx modulation of apoptosis (1, 19, 23, 42, 50, 53, 79, 91). HBx induced apoptosis in primary rat hepatocytes that were cotransfected with the I κ B superrepressor (I κ B-SR), an inhibitor of NF- κ B activity (Fig. 3), demonstrating that HBx inhibition of apoptosis requires activation of NF- κ B (13, 15, 28, 89). We also demonstrated that HBx expressed from HBV stimulates NF- κ B activity in primary rat hepatocytes (Fig. 7) and that transfection of primary rat hepatocytes with the I κ B-SR followed by AdHBV infection stimulated cytochrome *c* release (Fig. 8). Thus, HBx modulation of apoptosis is NF- κ B dependent in cultured primary rat hepatocytes both when HBx is expressed in the absence of other HBV proteins and in the context of HBV replication. HBx activation of NF- κ B inhibits spontaneous and TNF- α -induced apoptosis; however, when NF- κ B activity is inhibited, HBx induces apoptosis. These observations correlate with the results of our recently published work which demonstrated that the effect of HBx expression on mitochondrial membrane potential also varies depending on the status of NF- κ B (23) and previous studies that have linked HBx inhibition of apoptosis to activation of NF- κ B (81, 95). In addition, the results of our apoptotic assays in primary rat hepatocytes could explain some of the discrepancies in other published studies that analyzed HBx modulation of apoptosis; HBx may have various effects on apoptosis in different cell types, depending on the levels of NF- κ B in those cells and the extent of HBx-induced NF- κ B activation. For example, unlike primary hepatocytes, certain cell lines have low levels of NF- κ B, and therefore, there is no need to inhibit protein translation to observe the proapoptotic effects of various stimuli (80). A similar conclusion was reached from work on HBx regulation of apoptotic pathways in NIH 3T3 cells with elevated levels of myc expression and alterations in NF- κ B signaling (81).

We next determined how HBx induces apoptosis in the absence of NF- κ B activity. Because the results of several studies suggest that HBx can interact with components of the MPTP and regulate MPTP activities and mitochondrial membrane potential, we examined whether HBx regulation of apoptosis is MPTP dependent (9, 23, 55, 67, 78, 83). In the absence of NF- κ B activity, CSA treatment abrogated the HBx-dependent induction of apoptosis, suggesting that HBx induces apoptosis through regulation of the MPTP (Fig. 4). Similar results were obtained when HBx was expressed in the absence of other viral proteins and in the context of HBV replication (Fig. 8A). Overall, these studies show that HBx activation of apoptotic pathways involves modulation of the MPTP. Similarly, our previously published results demonstrated that, in the absence of NF- κ B activity, HBx induced mitochondrial membrane depolarization by regulating the MPTP (23). Because HBx modulation of the MPTP regulates both mitochondrial membrane depolarization and apoptosis and because mitochondrial membrane depolarization is often an upstream event in the apoptotic pathway (6, 39), our results suggest that HBx-induced mitochondrial membrane depolarization is linked to the downstream activation of apoptosis.

Although our analyses of the proapoptotic activities of HBx in cultured primary rat hepatocytes required that we first inhibit NF- κ B activity and suggest that HBx may be predominantly antiapoptotic when expressed in the liver, our studies of the proapoptotic activities of HBx may also have direct biological relevance. It is well-known that there is zonal variation of hepatocyte metabolic activities within the liver and that within different liver zones, hepatocytes can differentially express various transcription factors (31, 48, 49). It is therefore possible that in vivo, HBx can be both pro- or antiapoptotic in hepatocytes, depending on the zonal location of the HBV-infected hepatocytes. In addition, as hepatocytes undergo the process of transformation during progression to HCC, the expression of NF- κ B may change; in this context, HBx could transition from an antiapoptotic protein to a proapoptotic protein. This may also explain why HBx can sensitize some established liver cell lines to TNF- α -induced apoptosis; it has been suggested that this HBx-induced sensitization may be correlated with lower levels of NF- κ B in these cells (80). Finally, from a mechanistic perspective, inactivation of NF- κ B in HBx-expressing cultured primary rat hepatocytes facilitates a direct evaluation of the effect of HBx on the MPTP in the absence of confounding signals from NF- κ B and may be required to fully elucidate the impact of HBx expression on the MPTP and proapoptotic pathways. This is similar to studies with TNF- α where a molecular analysis of its proapoptotic activities often requires inhibition of TNF- α -induced activation of NF- κ B and the consequential stimulation of antiapoptotic pathways (3).

The results of studies described here, combined with previous studies of HBx modulation of cytosolic calcium levels and HBV replication, support the model for HBx modulation of HBV replication and apoptosis in normal hepatocytes that is outlined in Fig. 9 (9, 23, 55). We previously demonstrated that an important function of HBx that regulates HBV replication is modulation of cytosolic calcium levels and linked HBx-induced elevation of cytosolic calcium levels to HBx modulation of the MPTP (55). However, elevation of cytosolic calcium levels is also linked to the activation of apoptotic pathways, a

FIG. 9. HBx regulation of apoptosis in primary rat hepatocytes. See Discussion for explanation.

condition which may negatively impact hepatocyte survival and HBV replication (62). We propose that in HBV-infected hepatocytes, although HBx modulates the MPTP to increase cytosolic calcium levels, which is necessary for HBV replication, HBx also activates the NF- κ B pathway to inhibit the activation of apoptotic pathways that results from increased cytosolic calcium levels (62). In addition, HBx stimulation of antiapoptotic signals may also protect hepatocytes from cytokine-mediated activation of apoptosis that results from innate and adaptive immune responses to an HBV infection. The proapoptotic activity of HBx may be an unavoidable consequence of its elevation of cytosolic calcium and stimulation of HBV replication, and HBx activation of NF- κ B would be essential to counterbalance this proapoptotic effect, enhance hepatocyte survival, and facilitate HBV replication (Fig. 9). This dual activity of HBx is reminiscent of viral proteins that regulate replication of adenoviruses, papillomaviruses, and polyomavirus (5, 59, 60). For example, the large T antigen of simian virus 40 activates cell proliferation pathways to favor viral replication yet consequentially activates cellular apoptotic pathways. However, the large T antigen also inactivates the proapoptotic cellular response by inhibiting the activity of p53 (59). Similar types of pro- and antiapoptotic responses are induced by E1A and E1B proteins of adenoviruses and E6 and E7 proteins of papillomaviruses (5, 60).

HBx modulation of apoptotic pathways may also contribute to the development of HBV-associated HCC. Studies have shown that NF- κ B levels and activity can be increased in tumor tissue from patients with HBV- and/or HCV-associated HCC compared to nontumor tissue from HCC patients, suggesting that elevated NF- κ B may contribute to tumor progression in hepatocytes (82). In addition, studies conducted in multidrug resistance 2 (Mdr2)-knockout mice demonstrated that NF- κ B-dependent inhibition of apoptosis can promote liver tumor formation, and inhibition of NF- κ B activity in these mice diminished tumor formation (66). In the context of HBV replication or in HBV-associated HCC where HBx is expressed in the absence of other viral proteins or HBV replication, HBx activation of NF- κ B could inhibit hepatocyte apoptosis and contribute to hepatocarcinogenesis. Conversely, it is possible that NF- κ B activity fluctuates during the course of an HBV infection, potentially as hepatocytes are regenerating, reacting to cytokines secreted by immune cells, or responding to viral

infection by activating innate intracellular antiviral pathways. In this context, HBx could alternate between induction and inhibition of apoptosis, and in the absence of activated NF- κ B, the ability of HBx to modulate the MPTP to increase cytosolic calcium levels and activate apoptotic pathways could contribute to hepatocarcinogenesis. Paradoxically, NF- κ B inactivation also promotes carcinogenesis in hepatocytes (52), and mice lacking I κ B kinase β expression develop higher numbers of liver tumors than wild-type mice do. This hepatocarcinogenesis is a direct consequence of the regenerative capacity of the liver; repeated cycles of apoptosis and proliferation are thought to facilitate the selection of hepatocytes that are resistant to apoptotic signals and to contribute to the development of HCC. The notion that apoptosis and regeneration may contribute to HBx-associated HCC is supported by studies conducted in an HBx transgenic mouse model (40). In these mice, prior to 12 months of age, rapid hepatocyte turnover was linked to increased hepatocyte proliferation and apoptosis, although it was not clear whether increased proliferation induced apoptosis or whether increased apoptosis induced regeneration (40). Interestingly, after 12 months of age, the HBx transgenic mice no longer displayed increased hepatocyte apoptosis, resulting in the development of HCC (40). During HBV infection in the liver, HBx could regulate similar proliferation and apoptotic pathways, eventually contributing to hepatocarcinogenesis. Finally, the ability of HBx to modulate apoptotic pathways and HBV replication might not initially contribute to carcinogenesis but rather promote its progression. In the context of a chronic HBV infection and the resultant inflammatory and regenerative response, rapid hepatocyte turnover may alter hepatocyte characteristics and initiate transformation; in this context, the modulation of apoptotic pathways may contribute to either the survival of transformed hepatocytes when the antiapoptotic activity of HBx dominates or the selection of hepatocytes that are resistant to apoptotic signals when the proapoptotic activity of HBx is dominant.

In summary, our studies in cultured primary rat hepatocytes have demonstrated that HBx can be either pro- or antiapoptotic, depending on the status of NF- κ B, and have defined HBx modulation of the MPTP and HBx activation of NF- κ B as essential for its pro- or antiapoptotic activities. Future studies will address the mechanisms associated with HBx regulation of the MPTP or activation of NF- κ B in cultured primary rat hepatocytes. Of particular interest is the mechanism associated with HBx modulation of NF- κ B, as our studies have shown that this HBx function controls its pro- or antiapoptotic activity in hepatocytes. There are conflicting studies regarding a potential role for protein kinase C in HBx regulation of NF- κ B activity, and the results of one study have provided compelling evidence that HBx-induced elevation of reactive oxygen species activates NF- κ B in HepG2 cells (91). Whether activation of protein kinase C or elevated levels of reactive oxygen species are required for HBx activation of NF- κ B in normal hepatocytes is unknown and an area that we are currently investigating. Future studies will also identify the specific factors that are activated by HBx pro- and antiapoptotic signals, the consequence of this for HBV replication, and the impact of these factors on the development of HBV-associated HCC.

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