Hepatitis B virus X protein targets Bcl-2 proteins to increase intracellular calcium, required for virus replication and cell death induction

Xin Genga,1, Chenghao Huangb,1, Yan Qinc, Janet E. McCombsc, Quan Yuanb, Brian L. Harrya, Amy E. Palmerc, Ning-Shiao Xiaa,2, and Ding Xuea,2

*Department of Molecular, Cellular, and Developmental Biology and 2Department of Chemistry and Biochemistry and BioFrontiers Institute, University of Colorado, Boulder, CO 80309; and 3National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health, Xiamen University, Xiamen, Fujian 361005, China

Edited* by Xiaodong Wang, University of Texas Southwestern Medical Center, Dallas, TX, and approved September 5, 2012 (received for review March 18, 2012)

Infection with the hepatitis B virus (HBV) promotes the development of hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) and is a leading cause of morbidity and mortality worldwide. HBV X protein (HBx) is an important effector for HBV pathogenesis, but its cellular targets and acting mechanisms remain elusive. We show here that HBx interacts with the anti-apoptotic proteins Bcl-2 and Bcl-xL through a Bcl-2 homology 3 (BH3)-like motif in mammalian cells. Importantly, mutations in the BH3-like motif that prevent HBx binding to Bcl-2 and Bcl-xL abrogate cytosolic calcium elevation and cell death induced by HBx expression in hepatocytes and severely impair HBV viral replication, which can be substantially rescued by restoring cytosolic calcium. These results suggest that HBx binding to Bcl-2 family members and subsequent elevation of cytosolic calcium are important for HBV viral replication. Consistently, RNAi knockdown of Bcl-2 or Bcl-xL results in reduced calcium elevation by HBx and decreased viral replication in hepatocytes. Our results suggest that HBx targets Bcl-2 proteins through its BH3-like motif to promote cytosolic calcium elevation, cell death, and viral replication during HBV pathogenesis, which presents an excellent therapeutic intervention point in treating patients with chronic HBV.

calcium signaling | apoptosis | necrosis

Hepatitis B virus (HBV) is a hepatocyte-specific DNA virus, which encodes several different viral proteins, including DNA polymerase, surface antigen, core antigen, and the X protein (HBx) (1, 2). Whereas the functions of the other viral genes in HBV DNA replication and virion assembly are better understood, the roles and mechanisms of HBx in HBV infection and pathogenesis remain enigmatic. HBx has been implicated in mediating multiple viral and cellular events in HBV-infected cells, including viral replication, transactivation of transcription factors, signal transduction, cell-cycle progression, and cell death (1, 2). Although HBx is found in both the cytoplasm and the nucleus, mitochondria appear to be an important site for HBx action, because expression of HBx has been shown to induce aggregation of mitochondria, loss of mitochondrial membrane potential, and cytochrome c release (3–6). How HBx interacts with mitochondria to cause these changes is not understood.

HBx is critical for viral pathogenesis and oncogenesis in HBV-infected livers (1, 2, 7). The HBx gene is the most frequently integrated viral sequence in hepatocellular carcinoma (HCC) and HBx protein is detected in most patients with HBV-related HCC, even in the absence of viral DNA replication (1, 8–10). In addition, HBV variants carrying mutations in HBx have been identified in HCC tissues and result in the loss of HBx-dependent activities, suggesting that evolving HBx functions may underlie HBV-related liver disease (11, 12). Moreover, HBx promotes liver tumorigenesis in transgenic mice lacking the other components of the HBV virion (13–17). Therefore, HBx plays an important role in the development of HBV-related HCC.

Calcium signaling is critical for multiple HBx activities (2, 18). HBx-induced elevation of cytosolic calcium has been shown to be important for HBV DNA replication, HBV core assembly, and activation of several transcriptional events and signaling cascades (19–24). Induction of apoptosis or necrosis by HBx also requires increased cytosolic calcium and mitochondria permeability transition (MPT), a process by which mitochondria regulate cellular calcium during homeostasis and cell death (25). However, the cellular targets with which HBx interacts to induce MPT and cytosolic calcium increase have not been identified. Many proteins were found to interact with HBx in various in vitro systems (1, 7, 18). However, most of these protein interactions have not been confirmed in conditions that recapitulate HBV infection in hepatocytes. Genetic redundancy of complex mammalian systems has been a major hurdle to definitive identification of HBx cellular targets. Using a simple, genetically tractable Caenorhabditis elegans animal model, we found that HBx interacts directly with the Bcl-2 homolog, CED-9, to induce cytosolic calcium increase and cell death, mimicking two important events downstream of HBx expression in hepatocytes (companion article, ref. 26). Here we demonstrate that HBx interacts with two Bcl-2 family members, Bcl-2 and Bcl-xL, in hepatocytes to induce cytosolic calcium elevation, cell death, and viral DNA replication. Therefore, Bcl-2 proteins are key cellular targets of HBx during HBV pathogenesis and may prove to be an effective point of pharmacologic intervention for the treatment of HBV-related liver disorders.

Results

HBX Binds Bcl-2 and Bcl-xL in Human Hepatocytes Through Its Bcl-2 Homology 3-Like Motif. Our analyses of HBx activities in C. elegans have suggested that HBx interacts with CED-9 in C. elegans to induce cytosolic calcium increase and cell death. Moreover, HBx can interact with human Bcl-2 and Bcl-xL in vitro through its Bcl-2 homology 3 (BH3)-like motif and this interaction is disrupted by two substitutions in conserved residues of the BH3-like motif (G124L and I127A) (26). We therefore investigated whether...
HBx interacts with Bcl-2 and Bcl-xL in human hepatic HepG2 cells by coimmunoprecipitation assay. In HepG2 cells transfected with pcDNA3.1-Flag-HBx, endogenous Bcl-2 and Bcl-xL, but not the anti-apoptotic Bcl-2 family member Mcl-1, were coprecipitated with Flag-HBx, using an antibody to the Flag epitope (Fig. 1A, lane 3). In contrast, no Bcl-xL and only a trace amount of Bcl-2 were coprecipitated with Flag-HBx(G124L, I127A) (Fig. 1A, lane 4), which was expressed in HepG2 cells at a comparable level to Flag-HBx (Fig. 1A, lanes 1 and 2). These results indicate that HBx associates with Bcl-2 and Bcl-xL in human cells through its BH3-like motif. Importantly, in HepG2 cells transfected with a 140% head-to-tail DNA copy of the HBV genome (pHBV) with or without HBx(G124L, I127A) mutations, endogenous Bcl-2 and Bcl-xL, but not Mcl-1, were coprecipitated with HBx, but not with HBx(G124L, I127A), using an antibody to HBx (Fig. 1B, lanes 3 and 4). Therefore, HBx expressed from its native promoter in a replicating HBV genome associates with endogenous Bcl-2 and Bcl-xL in human hepatic cells through its BH3-like motif.

HBx Induces Cell Killing and Cytosolic Calcium Increase in Hepatocytes Through its BH3-Like Motif. We analyzed the cell-killing activity of HBx in human cells by staining HBx-transfected HepG2 cells with Annexin-V Pacific Blue and propidium iodide (PI) to distinguish living cells from apoptotic and necrotic cells. Flow cytometry analysis of cells transfected with pcDNA3.1-Flag-HBx showed 10.6% apoptotic cells (Annexin-V positive and PI negative) and 7.9% necrotic cells (Annexin-V positive and PI positive) (Fig. 2B). Significantly less cell death was observed in HepG2 cells transfected with the same amount of pcDNA3.1-Flag-HBx(G124L, I127A) (4.87% apoptotic cells and 1.14% necrotic cells; Fig. 2C) or empty pcDNA3.1 vector (1.14% apoptotic cells and 0.32% necrotic cells; Fig. 2D). Therefore, as in C. elegans, HBx uses its BH3-like motif to bind anti-apoptotic Bcl-2 and Bcl-xL proteins and induces both apoptosis and necrosis in human hepatocytes.

Because calcium signaling is an important event downstream of HBx, we examined whether the BH3-like motif of HBx is necessary for HBx-induced elevation of cytosolic calcium. Cytosolic calcium in HepG2 cells transfected with pcDNA3.1-Flag-HBx or pcDNA3.1-Flag-HBx(G124L, I127A) was determined using the ratiometric fluorescent calcium indicator Fura-2 (27). We found that the resting calcium concentration was significantly increased upon expression of HBx compared with the vector-only control (Fig. 1C), whereas expression of HBx(G124L, I127A) failed to do so. The HBx-induced calcium elevation is not due to increased cell death, because a similar level of calcium increase was observed in the presence of Z-VAD, a pan-caspase inhibitor that blocks cell death (Fig. S1A). Therefore, HBx can induce an increase in cytosolic calcium that is dependent on its BH3-like motif and association with Bcl-2 family proteins.

**HBX-LIKE MOtif OF HBx Is Critical For HBV Viral Replication.** Given the critical role of HBx in HBV DNA replication (20), we examined whether interactions between HBx and Bcl-2 proteins are important for HBV replication. Cytoplasmic viral core particles, where HBV DNA replication occurs, were isolated from HepG2 cells transfected with the pHBV replicon with or without the HBx(G124L, I127A) mutations. The level of HBV DNA replication was examined by Southern blot analysis. Compared with cells transfected with wild-type HBV, HBV DNA replication was significantly reduced in cells transfected with the mutant pHBV (Fig. 3A). The level of the HBV core protein (HBcAg), which correlates with the level of HBV DNA (28), was also greatly reduced in cells transfected with the mutant HBV genome (Fig. 3B). Northern blot analysis showed no reduction in HBV pregenomic (pg)/precore (pc) RNA, preS/S mRNA, or HBx mRNA in cells transfected with the mutant pHBV replicon (Fig. 3C). Quantitative real-time PCR (Q-PCR) analysis of isolated viral particles revealed an eight- to ninefold reduction in HBV DNA replication in cells transfected with the mutant HBV genome compared with cells transfected with the wild-type HBV genome (Fig. 3D). These results indicate that the BH3-like motif of HBx is critical for HBV DNA replication but dispensable for HBV transcription. Importantly, HBV DNA replication in cells...
transfected with the mutant HBV genome was largely rescued by treatment with 5 μM ionomycin (Fig. 3D), an ionophore that increases cytosolic calcium (29). This result suggests that increased cytosolic calcium is an important signaling event downstream of HBx interaction with Bcl-2 proteins that stimulates HBV DNA replication.

We next examined the importance of HBx interaction with Bcl-2 proteins for HBV replication in an established mouse model of chronic HBV infection (30). The pHBV replicon with wild-type HBx or HBx(G124L, I127A) was introduced into BALB/C mice (n = 6) through hydrodynamic tail vein injection, along with a pcDNA3-GFP reporter as a control for injection efficiency. Cytoplasmic viral core particles were isolated from the liver 2 d after injection and subjected to Southern blot analysis (Fig. 4A). The average level of replicative DNA intermediates in livers of mice receiving the mutant pHBV replicon was reduced by two- to threefold compared with that of mice receiving the wild-type pHBV replicon (Fig. 4B). Expression of intracellular HBV core antigen (HBCAg) was similarly reduced in mice receiving the mutant pHBV replicon (Fig. 4C). These results confirm the importance of the BH3-like motif of HBx, and thus the association of HBx with Bcl-2 family proteins, for HBV DNA replication in HBV-infected liver.

**Bcl-2 and Bcl-xL Are Important for HBx-Induced Cytosolic Calcium Elevation and HBV Viral Replication in Hepatocytes.** We examined the importance of Bcl-2 proteins for HBV replication by knocking down the expression of Bcl-2 or Bcl-xL in HepG2 cells through RNA interference (RNAi). Compared with control short hairpin RNA (shRNA), Bcl-2 and Bcl-xL shRNA significantly reduced the expression of Bcl-2 and Bcl-xL in HepG2 cells, respectively (Fig. 5A and B). Importantly, Q-PCR analysis of isolated viral particles revealed a 21–41% reduction in HBV DNA replication in cells infected by lentivirus expressing Bcl-2 or Bcl-xL shRNA, compared with cells with control shRNA (Fig. 5A and B). Overexpression of the anti-apoptotic Mcl-1 protein, which does not interact with HBx (Fig. 1A and B), in cells treated with Bcl-2 or Bcl-xL shRNA did not prevent reduction of HBV DNA replication (Fig. S2), indicating that decreased HBV DNA replication caused by loss of Bcl-2 or Bcl-xL is unlikely due to impaired survival of the host cells. Moreover, RNAi knockdown of Bcl-2 or Bcl-xL dampened but did not obliterate intracellular calcium increase induced by HBx (Fig. S1 B and C), which is consistent with the finding that Bcl-2 or Bcl-xL knockdown reduced but did not block HBV DNA replication and indicates that Bcl-2 and Bcl-xL are partially redundant in mediating HBx functions. Bcl-2 and Bcl-xL double-knockdown cells were not viable for analysis of HBV viral replication and intracellular calcium changes. These results indicate that Bcl-2 and Bcl-xL are important for HBV viral replication and, together with the findings described above, provide strong evidence that HBx targets both Bcl-2 and Bcl-xL to increase intracellular calcium and to promote HBV DNA replication (Fig. 5C).

**Discussion**

Despite the critical role of HBx in HBV pathogenesis and oncogenesis, identification of HBx host targets has remained a major challenge in the last three decades (1, 2, 7). The intricacy of HBx activities, the lack of a powerful animal model to study HBV infection, variability among cell culture assays, and the complexity of the mammalian genome, which encodes at least six Bcl-x family members, have all contributed to the longstanding questions regarding the functions of HBx, its interactions with host targets, and its mechanisms of action. We have engineered a C. elegans animal model to identify HBx targets and downstream signaling pathways (26). Mimicking the initial cellular events that unfold following liver infection by HBV (17, 31, 32), HBx induces both apoptosis and necrosis in C. elegans through canonical cell death pathways. Interestingly, a unique gain-of-function mutation (G169E) in the Bcl-2 homolog CED-9, which inhibits cell death in C. elegans by blocking the binding of the endogenous BH3-only cell death inducer EGL-1 to CED-9 (33), also completely blocks the interaction between CED-9 and the BH3-like motif of HBx and HBx-induced cell death in C. elegans. Remarkably, Bcl-2 can fully substitute for CED-9 in C. elegans to mediate HBx-induced cell killing, indicating that Bcl-2 likely interacts with HBx in mammals. Indeed, we demonstrate here that HBx associates with Bcl-2 and Bcl-xL in human hepatocytes

![Fig. 2. HBx induces cell killing in human hepatic cells through its BH3-like motif. HepG2 cells were transfected with 1 μg of empty pcDNA3.1 vector (A), pcDNA3.1-Flag-HBx (B), or pcDNA3.1-Flag-HBx(G124L, I127A) (C), using pEGFP-C1 (1 μg) as a cotransfection marker. Only the HBx-transfected cells (GFP positive) are shown (Materials and Methods). Lower Left square indicates the percentage of living cells. Lower Right square indicates the percentage of apoptotic cells, and Upper Right square indicates the percentage of necrotic cells.](image-url)
through its BH3-like motif and that this protein interaction is crucial for HBx-induced cytosolic calcium elevation, cell death, and viral DNA replication. These findings suggest that molecular mimicry of endogenous BH3-only proteins by HBx enables its interactions with conserved host targets and hijacking of cell signaling pathways to benefit viral infection.

Calcium signaling is a critical event downstream of HBx expression that promotes HBV replication, core assembly, cell death, and other HBx functions (2, 20, 21, 25). HBx has been proposed to effect MPT (4–6, 20), which is important for intracellular calcium homeostasis and cell death (34, 35). Importantly, both Bcl-2 and Bcl-xL are mitochondrial proteins and have been implicated in regulating MPT (34, 36). HBx binding to Bcl-2 and Bcl-xL is critical for calcium regulation by HBx, because expression of HBx, but not HBx(G124L, I127A), which fails to bind Bcl-2 and Bcl-xL, triggers elevation of cytosolic calcium in hepatocytes. The finding that G124L/I127A mutations in the BH3-like motif of HBx greatly reduce HBV DNA replication in human and mouse hepatocytes, which can be substantially rescued by restoring cytosolic calcium with ionomycin, and the observation that RNAi knockdown of either Bcl-2 or Bcl-xL significantly compromises HBx-induced intracellular

**Fig. 3.** Interaction between HBx and Bcl-2 proteins is critical for HBV DNA replication in human hepatic cells. (A) Southern blot analysis of HBV DNA replication in HepG2 cells transfected with the wild-type or mutant (G124L, I127A) HBV replicon 3 d posttransfection. rc/ds DNA represents relaxed circular DNA and double-stranded DNA. ssDNA represents single-stranded DNA. (B) Measurement of the levels of the HBV core protein (HBcAg) in transfected HepG2 cells. The difference between cells transfected with the wild-type and mutant (G124L, I127A) HBV is shown as fold change to wild type. The data represent mean ± SD from three independent experiments. ***P < 0.0001. (C) Northern blot analysis of HBV transcription in HepG2 cells transfected with the wild-type or mutant HBV replicon 3 d posttransfection. Different HBV mRNAs are indicated. GFP mRNA from a cotransfection marker and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used as controls. (D) Quantitative PCR analysis of HBV DNA replication in HepG2 cells transfected with the wild-type or mutant HBV replicon with or without 5 μM ionomycin treatment. Three days posttransfection, cytoplasmic HBV viral particles were isolated and the viral DNA replication intermediates were quantified by real-time PCR (Materials and Methods). The results represent the fold change of the replicative intermediates from the mutant HBV replicon compared with those from the wild-type HBV replicon in HepG2 cells, using two different primer sets (one specific to the HBS ORF and one specific to the polymerase ORF). Data are presented as mean ± SEM. At least three independent experiments were performed for each dataset. ***P < 0.0001.

**Fig. 4.** Interaction between HBx and Bcl-2 proteins is critical for HBV DNA replication in mouse hepatocytes. (A) Southern blot analysis of HBV DNA replication in mouse livers after hydrodynamical injection of the wild-type or the mutant (G124L, I127A) HBV replicon 2 d postinjection. pcDNA3-GFP was coinjected as an injection marker. Equivalent amounts of liver tissues from six BALB/C mice in each injection group were collected and subjected to HBV DNA and HBcAg analyses (Materials and Methods). The levels of GFP and β-tubulin were determined by immunoblotting (IB) and used as controls to normalize the transfection efficiency. (B) Quantification of viral DNA intermediates from the Southern blot analysis in A. The results represent the relative value of different HBV DNA intermediates to the GFP control. The amounts of DNA and GFP were quantified by Quality One software (Bio-Rad). Data are presented as mean ± SEM. **P < 0.01. (C) A plot showing the levels of the HBV core protein (HBcAg) in mouse livers. All mice (n = 6) from each injection group were subjected to HBcAg analysis (Materials and Methods). RLU represents relative luminescence units (mean ± SEM). **P < 0.01.
calcium increase and HBV replication provide further confirmation that HBx targets Bcl-2 proteins to trigger cytosolic calcium elevation required for HBV replication and other events such as cell death (Fig. 5C).

Hepatocarcinogenesis is a complex and poorly understood process. Chronic hepatocyte cell death induced by HBV infection or carcinogens may trigger cycles of inflammation, immune response, and compensatory tissue regeneration and the acquisition of oncogenic mutations that lead to development of HCC (17, 31, 37). On the other hand, hepatocyte expression of prosurvival factors, such as Bcl-2 and p38α kinase, has been shown to be effective in preventing HCC development (37–39). Moreover, HBV viral replication plays an important contributing role in hepatocarcinogenesis. The development and progression of HCC in patients with chronic HBV strongly correlate with the viral DNA level in a dose-dependent manner (40, 41). Therefore, blocking HBV viral replication and HBV-induced cell death represents an effective strategy to treat patients with chronic HBV and to prevent the development of HCC. Our study suggests that the BH3-like motif of HBx is necessary for HBx binding to Bcl-2 family proteins, which results in elevated cytosolic calcium, efficient viral replication, and HBV-induced cell death. Therefore, therapeutically targeting the BH3-like motif of HBx could be a unique and effective strategy to treat patients with chronic HBV and to prevent development of HCC.

Materials and Methods

Immunoprecipitation Assays. HepG2 cells transfected with the pCDNA3.1-Flag-HBx constructs or the pHBV replicons (wild-type and G124L/I127A mutations) were lysed and precipitated using an anti-Flag antibody or an anti-HBx antibody. The proteins pulled down with HBx were detected by immunoblotting analysis.

Calcium Imaging and Analysis. HepG2 cells cotransfected with pcDNA3-mCherry and pcDNA3.1-Flag-HBx constructs (wild-type or G124L/I127A mutations) were incubated for 35 min with 4 µM Fura-2-AM and 0.04% Pluronics solution 48 h posttransfection, washed three times with buffer, and incubated for an additional 15 min to allow for cleavage of the acetoxymethyl (AM) ester, which trapped Fura-2 in the cells. Data were collected using the Metafluor software and analyzed by Excel (27). Statistical analysis was performed using a t test in the KaleidaGraph program. The error bars indicate SEM.

Quantification of HBV DNA Replication and HBCAg. Southern hybridization analysis and quantitative real-time PCR were used to quantify the amount of HBV replication DNA intermediates isolated from HepG2 cells or from mouse livers. The level of cytoplasmic HBCAg was measured by chemiluminescence, using a commercial assay kit.

Hydrodynamic Injection. Thirty micrograms of the pHBV replicon and 3 µg of pcDNA2-GFP were injected into the tail veins of BALB/c mice within 5 s in a volume of PBS equivalent to 10% of the mouse body weight. Livers of the injected mice were assayed for HBCAg and viral DNA 2 d after injection (30).

Detailed methods are available in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank T. Blumenthal and R. Garcea for comments and suggestions. This work was supported by National Institutes of Health Grants F30 NS070596 (to B.L.H.); R01 GM059083, GM079097, and GM088241 (to D.X.); and GM084027 (to A.E.P.); grants from China National Science Foundation (30925030) and the National Scientific and Technological Major Project (2013ZX10002-002) (to N.-S.X.); and a Burroughs Wellcome Fund Award (to D.X.).


**Supporting Information**

**Geng et al. 10.1073/pnas.1204668109**

**SI Materials and Methods**

**Molecular Biology.** The hepatitis B virus (HBV) X protein (HBx) cDNA clone was kindly provided by Xiao-Kun Zhang (Burnham Institute for Medical Research, La Jolla, CA). Standard methods of cloning, sequencing, and PCR amplification were used. To make HBx mammalian expression constructs, a DNA fragment encoding Flag-HBx was amplified by PCR and subcloned into the pcDNA3.1 (+) vector via its Nhel and EcoRV sites. The HBx mutant constructs containing G124L and I127A substitutions were generated using a QuickChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. The pHBV replicon contains a 140% DNA copy of the HBV genome and replicates in an HBx-dependent manner in HepG2 cells. pHBV containing HBx(G124L, I127A) was made by Quick-Change site-directed mutagenesis and confirmed by DNA sequencing.

**Cell Culture.** Human HepG2 cells were grown in DMEM with 10% (vol/vol) FBS (Sigma-Aldrich). Transfection of HepG2 cells was carried out using Effectene Transfection Reagent (Qiagen), following the manufacturer’s protocol. A transfection efficiency of 15–30% was routinely achieved. All transfection experiments were performed 24 h after plating. One microgram of pcDNA3.1, pcDNA3.1-Flag-HBx, or pcDNA3.1-Flag-HBx(G124L, I127A) was diluted in 100 µL of the DNA-condensation buffer (Buffer EC) supplied by the manufacturer (Qiagen). Eight microliters of enhancer and 10 µL of Effectene were sequentially added to the mixture, each followed by vortexing and incubation at room temperature per manufacturer’s protocol. After that, the mixture was supplemented with 0.6 mL complete medium and added to cells. Cotransfection of an enhanced GFP (EGFP)-expressing plasmid of pEFGP-C1 (1 µg) was included, where appropriate, to monitor transfection efficiency when performing flow cytometry analysis in the cell-killing assays.

**Coimmunoprecipitation Assays.** Coimmunoprecipitation experiments were performed using an antibody (M2) to the Flag epitope (Sigma) or an anti-HBx antibody (16F9). Briefly, HepG2 cells transfected with pcDNA3.1-Flag-HBx or pHBV constructs (wild type or mutant) were lysed in lysis buffer [100 mM NaCl, 0.5 mM MgCl2, 0.15 mM CaCl2, 1% (vol/vol) Nonidet P-40, 10 mM Tris-HCl, pH 8.0] containing protease inhibitor mixture tablets (Roche). Cell debris was removed by centrifugation at 10,000 x g for 10 min at 4 °C. The cell lysate was precleared with Protein G Sepharose beads (GE Healthcare) and subsequently incubated with the M2 or 16F9 antibody for 1 h with gentle shaking at 4 °C. Protein G Sepharose beads were then added and the incubation continued for another 2 h. The beads were washed five times with the lysis buffer. The bound proteins were resolved on a 15% SDS polyacrylamide gel and detected by immunoblotting, using anti-Bcl-2, anti-Bcl-xl, and anti-Mcl-1 antibodies (Cell Signaling Technology), respectively.

**Flow Cytometry.** Thirty-six hours posttransfection, living and dead HepG2 cells were scraped into the cell growth medium and precipitated by centrifugation. Cells from one well of a six-well plate (~6 x 10⁵ cells) were washed in cold PBS twice and resuspended in 600 µL Annexin V-binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4). Labeling of cells by propidium iodide (Sigma) and Annexin-V pacific blue (Invitrogen) was carried out according to the protocol provided by Invitrogen. Briefly, 100 µL of the suspended cells were transferred to a new tube. Five microliters of Annexin-V Pacific Blue (Invitrogen) and 5 µL of propidium iodide (Sigma) were added to each tube and incubated for 30 min at room temperature in the dark. Four hundred microliters of Annexin-V binding buffer were then added to each tube. Cells were analyzed on the CyAn ADP Analyzer (DakoCytomation) with background gates set to exclude non-transfected GFP(−) cells and to restrict Annexin-V Pacific Blue staining alone, or propidium iodide staining alone, to fewer than 0.5% positive events. Data were collected from more than 10,000 cells for each sample.

**Cytosolic Calcium Measurement.** HepG2 cells were cotransfected with pcDNA3-mcherry and pcDNA3.1-Flag-HBx constructs (wild type or mutant), using lipofectamine LTX (Invitrogen). After 48 h or later, cells were washed with HHBSS buffer ( Hank’s Balanced Salt Solution supplemented with 20 mM Hepes and 11 mM glucose) three times and then incubated with 4 µM Fura-2-AM and 0.04% Pluronic for 35 min. Cells were rinsed with HHBSS three times and left with HHBSS for another 15 min to allow for cleavage of the acetoxyethyl (AM) ester, trapping Fura-2 inside the cells.

Imaging experiments were performed on an Axiovert 200M inverted fluorescence microscope (Zeiss) with a Cascade 512B CCD camera (Roper Scientific), equipped with a Xenon Arc lamp (XBO75), 340/26 and 380/10 excitation filters, a 450-nm dichroic mirror, and a 535/45 emission filter. Excitation and emission filters were placed in filter wheels external to the microscope controlled by a Lambda 10-3 filter changer (Sutter Instruments) to allow for rapid acquisition of ratio images. Images were collected using Metafluor software (Universal Imaging). All images were collected on healthy cells with similar mCherry intensity, using a 40x oil objective, and were background corrected by generating a region of interest (ROI) on a blank area of the coverslip and subtracting the fluorescence intensity of each excitation channel. The background-corrected intensities at 340 nM and 380 nM excitation were used to calculate the Fura-2 340/380 ratio.

To determine the resting [Ca²⁺] in the cytosol, cells were treated with 5 µM ionomycin and 5 mM EGTA in Ca²⁺-free HBSS to obtain the ratio of the unbound indicator (R_min). Cells were then washed with Ca²⁺-free HBSS three times and treated with 5 µM ionomycin and 10 mM Ca²⁺ in HBSS to obtain the ratio of the calcium-saturated indicator (R_max). Ca²⁺ concentrations were calculated using the reported K_d value of Fura-2 and the experimentally derived R_min, R_max, R_min/S, and S (the emission intensity at 380 nm for Ca²⁺-free and Ca²⁺-bound Fura-2, respectively) in each individual cell according to the following equation: [Ca²⁺] = K_d x [(R - R_min) / (R_max - R)] x S / S. Details on the microscope, sensor calibration, and conversion of Fura-2 ratios into Ca²⁺ concentrations have been described previously (1). The results were presented as the fold increase of the calcium concentration in cells transfected with pcDNA3-Flag-HBx over that in cells transfected with the empty vector.

**Southern Blot Analysis.** HepG2 cells (3 x 10⁶ cell per sample) transfected with the pHBV replicon (wild type or mutant) were washed twice with cold PBS and lysed in 750 µL NET buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40] per 10-cm plate for 1 h. HepG2 cell lysates were clarified by centrifugation at 12,000 x g for 30 min at 4 °C. The supernatant was adjusted to 6 mM CaCl₂ and treated with 100 µg/mL of micrococcal nuclease for 30 min at 37 °C. The reaction was stopped by addition of EDTA to a final concentration of 25
mM. Proteins were digested with 0.2 mg/mL proteinase K and 0.5% SDS overnight at 37 °C. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation. After centrifugation at 12,000 × g for 30 min at 4 °C, the pellet was resuspended in 10 μL TE buffer. DNA samples were resolved on a 1.2% agarose gel, transferred to a nylon membrane (Bio-Rad), and hybridized with a digoxin-labeled DNA fragment covering the entire HBx gene.

Northern Blot Analysis. Total cellular RNA was extracted from transfected cells, using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Twenty micrograms of RNA was separated on a 1% denaturing formaldehyde agarose gel, transferred onto nylon membranes, and hybridized to probes covering the whole HBV genome, prepared as described above.

Quantitative Real-Time PCR Analysis. Intracellular HBV replicating intermediates were isolated from cytoplasmic viral core particles as described previously (2). The pHBV plasmid was used as the standard for quantification and serial dilutions from 2 × 10⁸ to 200 IU/mL were prepared. The primer sequences for quantitative PCR were chosen carefully from regions coding for HBV S antigen and polymerase. Sequences of primer set 1 (corresponding to HBs), forward primer, 5’ GTGTCTGCGGCGTTTTATCA 3’; reverse primer, 5’ GACAAAAGGGCAACATTCTT 3’; primer set 2 (corresponding to polymerase), forward primer, 5’ TACTAGTGCCATTTGTTCAGTGG 3’; reverse primer, 5’ CACGATGCTGTACAGACTTGG 3’; Real-time PCR analyses were performed using a SYBR Green Premix Ex Taq Kit (Takara Bio) in an ABI Prism 7500 PCR system (Applied Biosystems). PCR products were analyzed by fluorescence. Each standard dilution was subjected to two PCR runs in at least two independent experiments. Based on the mean threshold cycles (Cₜ) for each dilution, a linear regression was carried out with the Cₜ values as a function of the decadic logarithm of the number of template molecules per reaction. Least-squares regression analysis, performed by the ABI prism 7500, plotted Cₜ as a function of nominal input number. The measured raw copy number from each reaction was calculated using the Cₜ value of each PCR interpolated against the linear regression of the standard curve. Each DNA specimen was subjected to four PCR runs with at least three independent experiments.

HBV Core Antigen Analysis. The amounts of HBV core antigen (HBcAg) were determined by chemiluminescence, using a commercial assay kit (Wantai).

Mice. BALB/c mice (male, 6–7 wk old) were divided into two groups (six mice for each injection group). Thirty micrograms of the pHBV replicon (wild type or mutant) plus 3 μg of pcDNA3-GFP were injected into the tail veins of mice within 5 s in a volume of PBS equivalent to 10% of the mouse body weight. Livers of these mice were collected and assayed for the levels of HBcAg and viral DNA, respectively, 2 d after injection as described previously (3).

shRNA-Mediated Knockdown in HepG2 Cells. Validated Mission shRNA lentiviral particles targeting human Bcl-2 or the control shRNA lentiviral particles were obtained from the Functional Genomic Facility of the University of Colorado. We generated a short hairpin RNA targeting human Bcl-xL, using the lentivirus-based shRNA delivery vector pLL3.7-neo. Viral particles targeting Bcl-xL were produced in HEK 293FT cells and used to infect HepG2 cells. For shRNA knockdown experiments, cells were plated 24 h before infection. Lentiviral particles were added in the presence of hexadimethrine bromide at the recommended multiplicity of infection. Infected cells were selected in media with puromycin (1 μg/mL; Sigma-Aldrich) or neomycin (0.5 mg/mL; Sigma-Aldrich). The Bcl-2 mRNA sequence targeted by the shRNA is 5’ CCGGGAGATAGTGATGATC 3’. The Bcl-xL mRNA sequence targeted by the shRNA is 5’ GTGGAACTCATGGAAGATA 3’.

HBx-induced cytosolic calcium elevation is not due to increased cell death and is dependent on the normal expression of Bcl-2 and Bcl-xL. (A) Z-VAD did not inhibit HBx-induced intracellular calcium increase. HepG2 cells cotransfected with 0.75 μg of pcdNA3-mCherry and 2.25 μg of pcdNA3.1-Flag-HBx or 2.25 μg of empty vector were untreated, treated with 0.025% DMSO, or treated with 5 μM of Z-VAD in 0.025% DMSO. Two days after transfection, cytoplasmic calcium concentrations were determined using the Fura-2-AM assay as described in Fig. 1C. The results shown are the fold increase of the calcium concentration in cells transfected with pcdNA3.1-Flag-HBx over that in cells transfected with the empty vector under the same drug treatment (untreated, DMSO treated, or Z-VAD treated, respectively). In all three conditions, expression of HBx induced significantly higher intracellular calcium than the vector-only control. Data are presented as mean ± SEM (n > 12). *P < 0.05; **P < 0.01. (B and C) (Upper) HepG2 cells infected by lentivirus expressing control, Bcl-2, or Bcl-xL shRNA were cotransfected with 0.75 μg of pcdNA3-mCherry and 2.25 μg of pcdNA3.1-Flag-HBx or 2.25 μg of empty vector. Two days after transfection, cytoplasmic calcium concentrations were determined. The results represent the fold increase of the calcium concentration in cells transfected with pcdNA3.1-Flag-HBx over that in cells transfected with the empty vector under the same RNAi treatment (control, Bcl-2, or Bcl-xL shRNA, respectively). Data are presented as mean ± SEM (n > 10). (Lower) shRNA knockdown efficiency and the expression of HBx were examined by immunoblotting using anti-Bcl-2, anti-Bcl-xL, and anti-Flag antibodies and β-actin as a loading control as described in Fig. 5. **P < 0.01; n.s., no significant difference (P > 0.05).
Fig. S2. Overexpression of Mcl-1 did not prevent reduction of HBV DNA replication caused by loss of Bcl-2 or Bcl-xL. (A and B) (Left) HepG2 cells infected by lentivirus expressing control, Bcl-2, or Bcl-xL shRNA were cotransfected with 2 μg of the pHBV replicon and 2 μg of pcDNA3-Flag-Mcl-1 and subjected to Q-PCR analysis as described in Fig. 3D. (Right) shRNA knockdown efficiency and the expression level of Flag-Mcl-1 were analyzed by immunoblotting using anti-Bcl-2, anti-Bcl-xL, and anti-Flag antibodies and β-actin as a loading control. Data are presented as mean ± SEM. **P < 0.01; ***P < 0.0001.