The Hepatitis B Virus X Protein Modulates Hepatocyte Proliferation Pathways To Stimulate Viral Replication

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Worldwide, there are over 350 million people who are chronically infected with the human hepatitis B virus (HBV); chronic HBV infections are associated with the development of hepatocellular carcinoma (HCC). The results of various studies suggest that the HBV X protein (HBx) has a role in the development of HBV-associated HCC. HBx can regulate numerous cellular signal transduction pathways, including those that modulate cell proliferation. Many previous studies that analyzed the impact of HBx on cell proliferation pathways were conducted using established or immortalized cell lines, and when HBx was expressed in the absence of HBV replication, and the precise effect of HBx on these pathways has often differed depending on experimental conditions. We have studied the effect of HBx on cell proliferation in cultured primary rat hepatocytes, a biologically relevant system. We demonstrate that HBx, both by itself and in the context of HBV replication, affected the levels and activities of various cell cycle-regulatory proteins to induce normally quiescent hepatocytes to enter the G\(_1\) phase of the cell cycle but not to proceed to S phase. We linked HBx regulation of cell proliferation to cytosolic calcium signaling and HBx stimulation of HBV replication. Cumulatively, our studies suggest that HBx induces normally quiescent hepatocytes to enter the G\(_1\) phase of the cell cycle and that this calcium-dependent HBx activity is required for HBV replication. These studies identify an essential function of HBx during HBV replication and a mechanism that may connect HBV infections to the development of HCC.

Viruses often encode proteins that modulate normal cellular processes to create an environment that facilitates viral replication. A potential consequence of the activities of viral proteins that alter normal cellular signaling pathways is that they can stimulate cell transformation and cancer progression. Included among confirmed oncogenic viruses is the hepatitis B virus (HBV); HBV is the prototype member of the Hepadnaviridae, a family of hepatotropic viruses (57). The HBV genome is a partially double-stranded, circular DNA that contains four overlapping open reading frames (ORFs) that encode the viral envelope, capsid, reverse transcriptase, and X (HBx) proteins (57). Worldwide, there are over 350 million people who are chronically infected with HBV; approximately 25\% of these chronically HBV-infected individuals develop hepatocellular carcinoma (HCC) (3, 57). While there is evidence of a strong link between chronic HBV infections and HCC development, exactly how chronic HBV infections can lead to the development of HCC has not been defined. The results of numerous studies suggest that the development of HBV-associated HCC involves a combination of the activities of HBV proteins that modulate cell signal transduction pathways, such as HBx, and the consequences of the host immune response to infection, including immune-mediated destruction of HBV-infected hepatocytes that induces repeated liver regeneration cycles.

HBx is a multifunctional protein that is expressed from the smallest ORF of HBV. HBx can regulate cellular signal transduction, transcription, protein degradation, and calcium signaling pathways and affect cellular processes such as apoptosis and cell proliferation (reviewed in references 13 and 57). Unfortunately, because many HBx activities have been analyzed using immortalized or transformed cell lines, often not of liver origin, a precise understanding of how HBx affects hepatocyte physiology and influences HBV replication has been confounded by discrepant HBx activities that have been observed in various experimental systems (reviewed in reference 13). HBx expression strongly influences HBV replication, although whether HBx is required for HBV replication is still debated (9, 11, 31, 38, 44, 64). Additionally, whether HBx expression is directly or indirectly involved in the development of HCC has varied depending on the experimental system used for analysis. In HBx-transgenic mice, HBx expression either directly caused the formation of liver tumors or made mice more susceptible to chemical carcinogens, depending upon the genetic background of the mice (15, 32, 40, 63, 66, 71). Exactly how HBx might affect cancer progression in individuals with chronic HBV infection is not known.

The process of cell proliferation involves a series of organized events that can be separated into five phases; quiescent cells are in G\(_0\) phase, cells that have entered the cell cycle and are preparing to replicate their DNA are in G\(_1\) phase, cells replicating their DNA are in S phase, cells that have completed replicating their DNA and are preparing to divide are in G\(_2\) phase, and cells that are dividing into daughter cells are in M (mitosis) phase (reviewed in references 22 and 26). The results of some studies have shown that cell cycle progression can be altered in cells that contain replicating HBV, and the status of cell proliferation pathways can also affect HBV replication in some experimental systems (reviewed in reference 41). Studies
that examined the effect of HBV replication on the cell cycle in Huh7 cells, a human hepatoma cell line, and primary marmoset hepatocytes demonstrated that in the context of HBV replication, these cells stall in G2 phase (17). The results of a different study demonstrated that entry into S phase was decreased in Huh7 cells and HepG2.2.15 cells, human hepatoblastoma HepG2 cells that contain an integrated HBV genome and can produce infectious HBV (23). Studies that analyzed the effect of cell cycle phase on HBV replication demonstrated that HBV replication levels in HepG2.2.15 cells were low in S phase but higher in G0/G1 phase (29). In another study of HepG2.2.15 cells, HBV replication levels were highest in G1 and G2 phases and lowest in S phase (46). Cumulatively, the results of studies that have analyzed the impact of HBV replication on cell proliferation and the effect of cell cycle progression on HBV replication suggest that cell proliferation pathways may be regulated by HBV to stimulate viral replication.

Most studies that have focused on the effect of HBx on cell proliferation were conducted when HBx was expressed in the absence of other HBV proteins; HBx was shown to either inhibit or induce cell cycle progression, depending on the exact conditions of the study (reviewed in reference 41). Studies of Chang liver cells, HepG2 cells, NIH 3T3 cells, and HL-7702 cells suggested that HBx can induce arrested cells to enter the cell cycle, enter the cell cycle but stall in S phase, or progress through the cell cycle more rapidly (4, 5, 10, 16, 34, 37, 72). HBx can also modulate the levels of p16, p21, p27, cyclin D1, cyclin A, and cyclin B1; activate the p21 promoter; and increase the activity of cyclin-dependent kinase 2 (CDK2) in various cell lines and primary hepatocytes, although the precise effects of HBx varied in different experimental conditions (2, 17, 25, 36, 37, 45, 47, 48, 50). Andrisani’s group has provided direct support for the notion that HBx can have different effects on cell proliferation pathways depending on specific cellular characteristics (37). This group generated two HBx-expressing liver cell lines that were derived from the same parental cell line; one of these cell lines displayed a phenotype consistent with that of a differentiated hepatocyte, whereas the other HBx-expressing cell line was more dedifferentiated. The dedifferentiated cells displayed HBx-dependent G1 and S phase entry but paused early in S phase. The differentiated hepatocytes displayed HBx-dependent G1, S, and G2/M phase progression; induction of cyclins D1, A, and B1; and activation of CDK1 (37). Overall, the results of various studies suggest that when HBx is expressed in the absence of other HBV proteins, it can modulate cellular proliferation pathways; however, whether HBx expression affects proliferation of normal hepatocytes when it is expressed in the context of HBV replication has not been addressed.

The in vivo effects of HBx on hepatocyte regeneration and cell cycle progression have been examined using HBx-transgenic mice. The results of two studies using HBx-transgenic mice demonstrated that HBx decreased hepatocyte regeneration after partial hepatectomy and caused a delay in the G1/S transition (67, 68). In contrast, hyperproliferation and increased apoptosis of hepatocytes was noted to occur in another HBx-transgenic mouse model (35). Recently, Hodgson et al. determined that in a different HBx-transgenic mouse model, HBx did not effect total hepatocyte division but did induce a fraction of hepatocytes to enter the cell cycle prematurely (28). In summary, studies that have analyzed the impact of HBx on cell cycle progression in HBx-transgenic mice demonstrated that when HBx is expressed in the absence of other HBV proteins, it can regulate hepatocyte proliferation pathways; however, the exact impact appears to vary in different mouse models.

To examine the specific effect of HBx on cell cycle progression, both when expressed in the absence of other HBV proteins and when expressed in the context of HBV replication, and to eliminate the variability associated with the use of transformed and immortalized cell lines, we analyzed the effect of HBx on cell proliferation pathways in cultured, primary rat hepatocytes. Primary rat hepatocytes are a biologically relevant model system used to study normal hepatocyte physiology and liver diseases (7, 18, 21, 30, 52). Our studies addressed the effect of HBx on the levels and activities of cell cycle-regulatory proteins and on overall cell cycle progression and linked HBx-induced modulation of cell proliferation pathways to cytosolic calcium signaling. Lastly, we demonstrated that modulation of hepatocyte proliferation pathways is an essential function of HBx that stimulates HBV replication, potentially linking this HBx activity to processes involved in the development of HBV-associated HCC.

MATERIALS AND METHODS

Isolation and maintenance of primary rat hepatocytes. Primary rat hepatocytes were isolated as previously described (58). The hepatocytes were plated on collagen-coated tissue culture plates at approximately 2.0 × 10⁶ cells/cm² plate (∼80% confluent) and maintained in Williams E medium supplemented with 0.2 mM l-glutamine, 0.1 mM sodium pyruvate, 0.4 μg/ml insulin-transferrin- selenium (ITS), 0.5 μg/ml hydrocortisone, 0.5 ng/ml epidermal growth factor (EGF), and 2% dimethyl sulfoxide (DMSO) at 37°C in 5% CO₂. The levels of growth factor used to supplement the medium was approximately one-tenth of what is used in normal primary hepatocyte medium to reduce the effect of growth factors on cell proliferation. Hepatocyte morphology and expression of hepatocyte-specific mRNAs were monitored throughout the time course of the experiments (see below). Animal surgery and hepatocyte isolation complied with all relevant federal and institutional policies.

Cells, transfections, and reagents. Rat adrenal medullary endothelial cells (RAMEC) (a gift from Peter Leikis, Drexel University [51]), were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 μg/ml gentamicin. Cells were maintained at 37°C in 5% CO₂.

Cultured, primary rat hepatocytes were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. All transfections were performed 24 h after plating. Human hepatocyte growth factor (HGF) was purchased from Sigma, propidium iodide was purchased from Molecular Probes, RNase A was purchased from Fisher Scientific, and 1,2-bis(o-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid-tetraacetoxymethyl ester (BAPTA-AM) was purchased from Invitrogen.

Antibodies. The anti-p15, anti-p27, and anti-cyclin D1 antibodies were from Cell Signaling; the anti-Flag-M2 antibody was from Stratagene; the anti-c-tubulin, anti-p16, anti-p21, anti-proliferating cell nuclear antigen (anti-PCNA), anti-cyclin E, anti-cyclin A, anti-CDK2, and anti-CDK4 antibodies were from Santa Cruz Biotechnology, Inc. The anti-β-actin antibody was from Sigma, and the anti-HBeAg antibody was from DakoCytomation, Inc.

Plasmids. FL1-154 HBxs, containing N-terminally Flag-tagged HBx cloned into the pcDNA3.1(−) vector, have previously been described (19). pGEMHBV (payw1.2) and pGEMHBV(HBx)− have previously been described (44, 55, 56). The p16 overexpression plasmid, containing the p16 sequence cloned into the pBABE vector, was a gift from Joan Brugge, Harvard University.

Recombinant adenoviruses. We have found that some analyses, such as HBV replication, require that 100% of cultured, primary rat hepatocytes express replicating HBV, and for studies involving HBV replication, we used HBV-expressing recombinant adenoviruses (20). These recombinant adenoviruses have previously been described (20). All the recombinant adenoviruses encode green
fluorescent protein (GFP). The recombinant adenoviruses used in this study are referred to as AdGFP, the control, GFP-only recombinant virus; AdGFP-HBx, which expresses GFP and HBx; AdHBV, which expresses GFP and HBV from a greater-than-unit-length cDNA of HBV; and AdHBV(HBx), which is identical to AdHBV but contains a mutation that prevents expression of HBx while not affecting expression from the overlapping polymerase ORF (20). GFP expression is used to monitor adenovirus infection efficiency.

Reverse transcriptase PCR (RT-PCR) analysis of hepatitis c mRNA. Albumin (ALB), transferrin (TFN), and hepatocyte nuclear factor 4 (HNF4) mRNAs were monitored as markers of differentiated hepatocytes (8, 53). We also examined two connexins, connexin 26 (CX26) and connexin 43 (CX43). Connexin 26 is present in rat hepatocytes, while connexin 43 is present only in liver sinusoidal endothelial, Kupffer, and stellate cells and served as a negative control and a means of establishing the purity of the cultured hepatocytes (49). Hepatocyte RNA was isolated using the TRIzol reagent according to the manufacturer’s protocol (Invitrogen). RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad) per the manufacturer’s instructions. Primers for the subsequent ALB, TNF, and HNF4 PCRs were previously described (19). The primers for connexin 26 and connexin 43 are as follows: the forward primer sequence for connexin 26 was 5'-GCAAGCTTGATTTGGGCACACTAC-3', and the reverse primer sequence was 5'-CGAATTCGGACTTCGAGGAAA TACCG-3'; the forward primer sequence for connexin 43 was 5'-GCAAGCTTGTG TCAAAGTGATGACAC-3', and the reverse primer sequence was 5'-CGGATCCGGACTTCGAGGAGAC-3'.

Propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis. Primary hepatocytes were plated at approximately 50% confluence. Twenty-four hours after infection with AdGFP or AdGFP-HBx (48 h after plating), hepatocytes were washed once with 1× phosphate-buffered saline (PBS), trypsin treated, and pelleted. For each experimental parameter, 1 × 10⁹ hepatocytes were resuspended in 500 µl of 1× PBS plus 0.1% dextrose and fixed using cold 70% ethanol for at least 24 h. Cells were then pelleted, resuspended in 400 µl propidium iodide staining solution (70 µM propidium iodide, 40 mM sodium citrate, and 0.5 mM RNAse A), and incubated at 37°C for 2 h. Cells were analyzed using a Guava Technologies flow cytometer and the Cell Cycle program according to the manufacturer’s instructions.

Cell collection and Western blot analysis. Twenty-four hours following transfection or recombinant adenovirus infection, cells were washed with 1× PBS, scraped into 1× PBS, and pelleted at 2,000 rpm for 3 min at 4°C. Cells were then lysed in 0.5% sodium dodecyl sulfate (SDS) lysis buffer (0.5% SDS, 240 mM Tris, pH 6.8, and 10% glycerol), and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad). Equal amounts of protein were loaded on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane (Bio-Rad) and blocked for 2 h in 5% milk in 1× PBS. After incubation with primary antibody overnight at 4°C, the membrane was washed with 1× Tris-buffered saline (TBS) plus 0.1% Tween 20, incubated with an Alexa Fluor-conjugated secondary antibody, and visualized using the quantitative Odyssey infrared imaging system (Licor Biosciences) according to the manufacturer’s instructions. Western analysis was conducted with antibodies specific for p15, p16, p21, p27, cyclin D1, cyclin E, cyclin A, PCNA, CDK4, CDK2, HbeAg, or Flag. Equal loading of protein was confirmed by analysis of α-tubulin. All protein levels were first normalized to α-tubulin levels, and then fold differences were determined by dividing the intensity of the protein in the FL1-154 HBx, AdHBV, or pGEMHBV lanes by the intensity of the protein in the pcDNA3.1(−), AdHBV(HBx−), or pGEMHBV(HBx−) lanes, respectively. Statistical significance of fold differences was determined using Student’s t test.

CDK activity assays. Immunoprecipitation of CDK4 complexes by use of antibodies specific for CDK4 was performed 24 h following transfection with pcDNA3.1(−) or FL1-154 HBx or infection with AdHBV or AdHBV(HBx−), as previously described (42). A purified, glutathione S-transferase (GST)-tagged, C-terminal fragment (amino acids 729 to 926) of the retinoblastoma protein (GST-Rb) was used as the substrate for CDK4 phosphorylation (the substrate was a gift from Peter Adams, The Beaton Institute for Cancer Research, Glasgow, Scotland) and purified as previously described (42). Immunoprecipitation of CDK2 complexes by use of antibodies specific for CDK2 was performed 24 h following transfection with pcDNA3.1(−) or FL1-154 HBx or infection with AdHBV or AdHBV(HBx−), as previously described (11). Histone H1 (Sigma) was used as the substrate for CDK2 phosphorylation.

HBV replication assay. Primary hepatocytes were infected with AdHBV and cotransfected with either pBABE control vector or p16 overexpression plasmid. At 72 h postinfection, HBV core particles were isolated, and HBV replication was analyzed via Southern blotting as previously described (11).

RESULTS

Confirmation of authentic, differentiated hepatocytes and HBx expression. The cultured, primary rat hepatocytes were monitored for maintenance of normal hepatocyte morphology and expression of specific markers of differentiated hepatocytes. To confirm that the hepatocytes maintained expression of differentiated hepatocyte-specific markers, RNA was isolated and reverse transcribed, and sequences of specific mRNAs were amplified using PCR. Albumin (ALB), transferrin (TFN), hepatocyte nuclear factor 4 (HNF4), and connexin 26 (CX26) were used as accepted markers of differentiated hepatocytes (8, 49, 53). In addition, expression of CX43, a marker present in dedifferentiated hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, and stellate cells was used to determine the purity of hepatocytes at the time of isolation and throughout the time course of our studies (49). Hepatocyte-specific markers were expressed immediately following isolation and in hepatocytes that were maintained in culture for 72 h, the time frame for our longest experiments (Fig. 1). Expression of connexin 43 was not detected in hepatocytes immediately following isolation or after 72 h in culture. RAMEC mRNA was used as a positive control for connexin 43 expression (51). The results of RT-PCR studies indicated that the cultured, primary rat hepatocytes maintained their differentiated status throughout the time course of our studies and contained undetectable levels of contamination with other liver cell types.

HBx blocks cell cycle progression of cultured, primary rat hepatocytes. Analysis of the expression of cell cycle proteins and overall cell cycle profiles sometimes requires that the cells are synchronized. In vivo, hepatocytes are normally quiescent, unless the liver is regenerating (65). We examined the cell cycle phase of freshly isolated hepatocytes and compared this to hepatocytes collected 12, 24, 36, 48, and 72 h following isolation and culture; hepatocytes were plated at approximately 80% confluence. Hepatocytes were fixed and stained with propidium iodide, and DNA content was analyzed via FACS analysis. We determined that hepatocytes collected through 72 h postisolation demonstrated cell cycle profiles that were identical to those of freshly isolated hepatocytes (unpublished observations). These profiles demonstrated that the majority (∼80%) of cells exhibited a DNA content indicative of G₁/G₀ phase but that ∼20% of cells had a G₂-phase content of DNA. Various serum starvation protocols and chemical inhibitor treatments were employed to reduce this G₂-phase peak in an attempt to completely synchronize the hepatocytes in G₁ phase; however, none of these treatments affected the G₂-phase peak (data not shown). A subset of hepatocytes are bineural (54); in these studies, bineucleated hepatocytes would accumulate in the G₂-phase DNA peak. When bineural hepatocytes were counted and compared to the levels for the FACS profiles, we determined that the percentage of bineural cells in our isolated hepatocytes was approximately equal to the percentage of cells within the G₂ phase peak of the cell cycle profile (data not shown). These observations suggest that hepatocytes plated at a confluence of 80% or higher maintain their quiescent phenotype; we also have noted that initial plating of rat hepatocytes at a confluence of 80% or higher facilitates long-term maintenance of these cells (T. L. Gearhart and...
cells. Cells were collected at 24 h post-hepatocyte growth factor (HGF; 10 ng/ml) and infected with recombinant adenoviruses. Initially, we observed no induction of cell proliferation of HBx-expressing or control hepatocytes when the cells were analyzed by propidium iodide staining and FACS (Table 1); these observations prompted us to question whether HBx might inhibit cell proliferation, which measures only relative DNA content, we could not determine if HBx was maintaining the cells in G0 phase or allowing progression to G1 phase while inhibiting progression beyond this point; the relative DNA contents of a cell are equivalent in G0 and G1 phases.

HBx can increase cell proliferation or induce cell cycle entry in some transformed and immortalized cell lines (reviewed in reference 41). We examined the effect of HBx on cell proliferation in cultured, primary rat hepatocytes that were infected with AdGFP-HBx or AdGFP recombinant adenoviruses. Initially, we observed no induction of cell proliferation of HBx-expressing or control hepatocytes when the cells were analyzed by propidium iodide staining and FACS (Table 1); these observations prompted us to question whether HBx might inhibit hepatocyte cell cycle progression. To address this, hepatocytes were plated at approximately 50% confluence, as opposed to the normal 80 to 90% confluence used in our other studies. Plating hepatocytes at a lower density generated an environment where normally quiescent, cultured hepatocytes could be stimulated to enter the cell cycle and replicate. Rat hepatocytes that were plated at 50% confluence were treated with hepatocyte growth factor (HGF; 10 ng/ml) and infected with AdGFP or AdGFP-HBx. Cells were collected at 24 h post-infection, fixed, and stained with propidium iodide, and the DNA content of the hepatocytes was analyzed by FACS. Hepatocytes that were infected with AdGFP and treated with HGF demonstrated a 13% increase in cells progressing into G2 phase in comparison to the untreated hepatocytes infected with AdGFP (P = 0.05) (Table 1, AdGFP alone versus AdGFP with HGF treatment). Importantly, HBx inhibited the HGF-stimulated increase of hepatocytes from progressing to G2 phase (Table 1, AdGFP-HBx with HGF treatment versus AdGFP with HGF treatment). These results indicate that HBx can inhibit HGF-stimulated hepatocytes from progressing to G2 phase. Due to the limitations of FACS analysis of cell proliferation, which measures only relative DNA content, we could not determine if HBx was maintaining the cells in G0 phase or allowing progression to G1 phase while inhibiting progression beyond this point; the relative DNA contents of a cell are equivalent in G0 and G1 phases.

HBx affects the levels of cell cycle proteins in primary rat hepatocytes. To determine whether HBx was pushing cells from G0 to G1 phase of the cell cycle, we compared the expression levels of various cell cycle proteins by Western blot analysis of proteins isolated from hepatocytes 24 h after transfection with HBx (FL1-154 HBx) or a vector control [pcDNA3.1(−)]. We first examined the levels of two members of the INK4 family of cell cycle inhibitors, p15 and p16, which prevent entry into and progression through the G1 phase of the cell cycle by inhibiting the cyclin D–CDK4/6 complex (62). We observed that HBx decreased the levels of both p15 and p16 in comparison to the level for control-transfected hepatocytes (Fig. 2B). The other major family of cell cycle inhibitors, the CIP/KIP family, includes p21 and p27. These proteins are active in G1 phase and assist in the activation of the G1 phase cyclin D–CDK4/6 complex but inhibit progression into S phase by binding and inhibiting the cyclin E–CDK2 complex (6, 62).

The levels of p21 and p27 were increased in HBx-expressing rat hepatocytes (Fig. 2C), which is consistent with previous observations for mouse hepatocytes (50).
Cyclins are proteins that help activate CDKs and are made and degraded according to the specific cell cycle phase in which they act. The primary active cyclin during G1 phase is cyclin D1, while cyclin E, cyclin A, and cyclin B act in late G1, S, and G2/M phases, respectively (reviewed in reference 26). We examined the levels of various cyclins and other cell cycle-activating proteins in cultured, primary rat hepatocytes transfected with either HBx (FL1-154 HBx) or a vector control [pcDNA3.1(–)]. HBx increased the levels of both G1 phase cyclins, cyclin D1 and cyclin E (Fig. 2C); however, the level of cyclin A was not affected (Fig. 2D). We also examined the level of an additional active S phase factor, PCNA. PCNA is a processivity factor required for DNA synthesis (reviewed in reference 26); HBx did not affect the level of PCNA (Fig. 2D). Taken together, these results demonstrate that in cultured, primary rat hepatocytes, HBx decreases the levels of proteins that prevent cell cycle entry and exit from G0 phase, increases the levels of essential G1 phase proteins, and increases the levels of proteins that block progression through S phase. These activities of HBx create a profile that is consistent with hepatocytes that have been stimulated to enter the cell cycle but stall in G1 phase.

HBx differentially affects the activities of the G1-phase cyclin-dependent kinases, CDK4 and CDK2. The results shown in Fig. 2 and Table 1 suggest that HBx induces normally quiescent hepatocytes to enter the cell cycle but stall in G1 phase. To support this conclusion, and to demonstrate a functional consequence for elevated cyclin D1 and cyclin E levels in HBx-expressing hepatocytes, we examined the activity of CDK4 and CDK2. During G1 phase, the cyclin D1-CDK4 complex is active and phosphorylates downstream targets, such as the retinoblastoma protein (Rb) (59–61). In its unphosphorylated state, Rb is bound to the S-phase-activating transcription factor E2F, thereby inhibiting progression into S phase. CDK4 was immunoprecipitated from HBx (FL1-154 HBx)- or vector control [pcDNA3.1(–)]-transfected hepatocytes, and CDK4 activity was analyzed using a standard kinase assay with a purified, GST-tagged, C-terminal fragment of Rb as the substrate (42). HBx increased CDK4 activity in comparison to the level for control-transfected primary hepatocytes (Fig. 3A). Thus, HBx increases the levels of G1-promoting proteins (Fig. 2) and activates CDK4 (Fig. 3A).

While cyclin E is also a G1-phase cyclin, it accumulates later in G1 phase, where it binds and helps activate CDK2; the activity of this complex is essential for the transition from G1 to S phase of the cell cycle (26). We observed an increase in cyclin E levels (Fig. 2C); however, we also observed an increase in the levels of p21 and p27, proteins that inhibit progression beyond G1 phase of the cell cycle by binding the cyclin E-CDK2 complex and repressing its activity. When we initially analyzed...
CDK2 activity in vector control or HBx-expressing hepatocytes that had been plated at 80% confluence, we observed almost undetectable levels of kinase activity (unpublished observation). Therefore, to confirm that HBx can stall hepatocyte proliferation in G1 phase, we used the same hepatocyte proliferation model as in our propidium iodide FACS studies. Hepatocytes were plated at approximately 50% confluence, and following transfection with HBx (FL1-154HBx) or a vector control [pcDNA3.1(−)]7, HGF was added to stimulate hepatocyte proliferation. CDK2 complexes were immunoprecipitated from HBx- or control-transfected hepatocytes at 24 h posttransfection, and CDK2 activity was assessed using histone H1 as the substrate. Control-transfected, HGF-treated hepatocytes demonstrated elevated levels of CDK2 activity, consistent with the results shown in Table 1, confirming that HGF can stimulate hepatocyte proliferation. HBxs significantly inhibited this increase in CDK2 activity (Fig. 3B). Contrary to what was observed with the increase in cyclin D1, the increase in cyclin E protein levels did not coincide with an increase in CDK2 activity; it is likely that the HBx-induced elevations of p21 and p27 levels inhibited the activity of cyclin E-CDK2 complexes. These observations are consistent with the notion that HBx induces cells to enter the cell cycle but stall in mid-G1 phase.

HBx that is expressed in the context of HBV replication modulates the levels and activities of cell cycle-regulatory proteins. We next examined whether HBx affected the levels of cell cycle-regulatory proteins when it was expressed in the context of HBV replication. While it is likely that similar HBx activities will be observed in the context of HBV replication, it is also possible that other HBV proteins could affect HBx regulation of cell cycle factors. Moreover, because many HBV-associated tumors continue to express HBx in the absence of other viral proteins or HBV replication (57), identifying HBx activities in the context of HBV replication and in the absence of other HBV proteins could help identify mechanisms of HBV-associated HCC. Due to the narrow host range of hepadnaviruses, rat hepatocytes cannot be directly infected with HBV. Therefore, we infected hepatocytes with replication-defective, recombinant adenoviruses expressing the HBV genome (AdHBV) or the HBV genome that is HBx deficient [AdHBV(HBx−)]. HBx is expressed at very low levels in the context of HBV replication and is difficult to detect unless large quantities of AdHBV-infected primary hepatocytes are combined and analyzed in a single Western blot (19). For this reason, it is impractical to perform all of the following experiments while simultaneously checking for HBx expression in every sample. We previously demonstrated that AdHBV facilitates HBV replication in primary rat hepatocytes and expresses HBx and that replication of HBV from AdHBV(HBx−) can be rescued by coinfection with AdGFP-HBx, confirming that replication in cultured, primary rat hepatocytes is HBx dependent and that this is the only defect of AdHBV(HBx−) (20). Cultured, primary rat hepatocytes that were infected with AdHBV or AdHBV(HBx−) were collected at 24 h postinfection, and the levels of various cell cycle-regulatory proteins were analyzed. We also monitored the level of the HBV core protein (HBeAg) as evidence that the levels of other HBV proteins were unchanged in AdHBV(HBx−)-infected hepatocytes in comparison to those in AdHBV-infected hepatocytes. Similar to results in HBx-transfected hepatocytes, AdHBV-infected hepatocytes had decreased levels of the cell cycle inhibitor proteins p15 and p16 in comparison to AdHBV(HBx−)-infected hepatocytes (Fig. 4A). AdHBV-infected hepatocytes also exhibited increased levels of the active G1-phase proteins p21, p27, cyclin D1, and cyclin E (Fig. 4B) and did not alter the levels of the S-phase proteins cyclin A and PCNA in comparison to the AdHBV(HBx−)-infected hepatocytes (Fig. 4C).

We also determined the effect of HBx that was expressed in the context of HBV replication on CDK4 and CDK2 activity. Primary rat hepatocytes were infected with either AdHBV or AdHBV(HBx−). CDK4 complexes were immunoprecipitated, and kinase activity was analyzed. We observed an increase in CDK4 activity in AdHBV-infected hepatocytes in comparison to the activity in AdHBV(HBx−)-infected hepatocytes (Fig. 4E). We also determined whether HBx that is expressed in the context of HBV replication affects CDK2 activity. Hepatocytes were plated at 50% confluence, infected with AdHBV or AdHBV(HBx−), stimulated with HGF, and then collected at 24 h postinfection. CDK2 complexes were immunoprecipitated, and CDK2 activity was analyzed. CDK2 activity was decreased in AdHBV-infected, HGF-treated hepatocytes in comparison to the level for AdHBV(HBx−)-infected, HGF-treated hepatocytes (Fig. 4F). Overall, the results of studies using AdHBV- and AdHBV(HBx−)-infected primary rat hepatocytes indicate that HBx regulates the levels of cell cycle-regulatory proteins when it is expressed in the absence of other HBV proteins and in the context of HBV replication and stimulates quiescent cells to enter but stall in G1 phase.

We have noted that for most studies that analyze the impact of HBx that is expressed in the context of HBV, 100% of cultured hepatocytes must be transduced with the HBV genome to detect the often subtle effects of HBx. However, to verify that the results of studies with the recombinant adenoviruses were a direct consequence of HBV HBx, we repeated some of our studies with pGEMHBV- or pGEMHBV(HBx−)-transfected primary rat hepatocytes (44, 55, 56); we focused on cell cycle proteins that consistently showed the greatest differences in HBx-expressing hepatocytes. As we previously reported, low levels of HBV replication can be detected in hepatocytes that are transfected with pGEMHBV, but higher levels of HBV replication are obtained with AdHBV-infected rat hepatocytes (20). When hepatocytes were transfected with pGEMHBV or pGEMHBV(HBx−), alterations in the levels of p16, p21, and cyclin A were similar to those observed in hepatocytes infected with AdHBV or AdHBV(HBx−) (Fig. 4D). These results demonstrate that the effects that we have observed with the recombinant adenoviruses are the direct consequence of HBx expression. In addition, the studies with pGEMHBV and pGEMHBV(HBx−) also show that the results of studies that used FL1-154HBx were not due to overexpression of HBx; in pGEMHBV, HBx is expressed from its endogenous promoter.

Modulation of cell cycle proteins by HBx and HBV requires cytosolic calcium signaling. Previous studies demonstrated that HBx can mobilize cytosolic calcium and that this HBx activity is required for HBV replication in HepG2 cells (11, 43); a similar dependence for calcium signaling during HBV replication has been observed in cultured, primary rat hepato-
cytoxes (T. L. Gearhart and M. J. Bouchard, unpublished observations). We next determined whether calcium signaling is required for HBx regulation of cell cycle progression. Primary rat hepatocytes were transfected with HBx (FL1-154 HBx) or a vector control [pcDNA3.1(H11002)] or infected with AdHBV or AdHBV[H11002] and treated with the intracellular calcium chelator BAPTA-AM (50 μM) for 18 h, and the levels of various cell cycle proteins were analyzed. We observed that the HBx- or AdHBV-induced decreases in p15 and p16 required cytosolic calcium signaling (Fig. 5A and 6A). In addition, we observed that the HBx- and AdHBV-induced increases in cyclin E, the cell cycle inhibitors p21 and p27, and CDK4 activity were also inhibited by BAPTA-AM treatment (Fig. 5B and C). These results demonstrate that HBx and HBV regulation of cell cycle-regulatory proteins requires cytosolic calcium signaling.

Cell cycle entry is required for HBV replication. The results of previous studies demonstrate that HBV replication may be affected by the status of cellular proliferation pathways (29, 46). Some previous studies examining the effect of cell cycle progression on HBV replication suggested that quiescent hepatocytes do not present an ideal environment for viral replication (23, 24); however, no studies have specifically examined the effect of quiescence on HBV replication. The levels of p16 must decrease for cells to exit G0, and overexpression of p16 can be used to block cyclin D1-CDK4/6 activation and prevent cell cycle entry (26, 62). Using a p16 overexpression plasmid, we sought to determine if entry into G1 phase and exit from G0 are required for HBV replication in cultured, primary rat hepatocytes. We confirmed increased expression of p16 and tested its ability to prevent exit from G0 by examining CDK4 activity, a marker of progression into G1 phase; overexpressed
p16 inhibited HBx-induced activation of CDK4 (Fig. 7A and B). To examine the effect of preventing progression from G₀ to G₁ phase on HBV replication, we first transfected hepatocytes with the p16 overexpression plasmid or the vector control and then infected the hepatocytes with AdHBV; transfection of DNA followed by infection with adenovirus increases transfection efficiency (70). Hepatocytes were collected at 72 h postinfection, and viral replication was analyzed. Inhibiting exit from G₀ and entry into G₁ phase by overexpressing p16 inhibited HBV replication (Fig. 7D). Importantly, overexpression of p16 did not affect the level of HBcAg, showing that decreased replication is likely not a consequence of p16 downregulating the expression of viral proteins (Fig. 7C). To our knowledge, this is the first direct evidence that quiescent hepatocytes must enter the cell cycle for HBV to replicate efficiently and identifies HBx regulation of cell cycle progression as an essential HBx activity that stimulates HBV replication.

DISCUSSION

Chronic infections with the hepatitis B virus (HBV) are associated with the development of hepatocellular carcinoma (HCC) (57). Activities of the HBV X protein (HBx) are thought to contribute to the development of HBV-associated HCC. HBx can interact with various cellular proteins, activate transcription pathways, regulate DNA repair pathways, elevate cytosolic calcium levels, and modulate protein degradation pathways (reviewed in references 11, 13, and 43). HBV is thought to encode two regulatory proteins, HBx and possibly the largest HBV envelope protein (13, 27). It is likely that HBx is the predominant HBV regulatory protein, and it is therefore not surprising that HBx can affect multiple cellular processes to stimulate HBV replication. Unfortunately, a potential consequence of the many activities of HBx is that some of these activities may alter normal hepatocyte physiology and contribute to hepatocyte transformation that is associated with chronic HBV infections.

While many previous studies have analyzed the impact of HBx expression or HBV replication on cell proliferation pathways (2, 4, 5, 10, 16, 17, 23–25, 28, 29, 34–37, 45–48, 50, 67, 68, 72), ours are significant for the following reasons. First, most previous studies that analyzed the effect of HBx on the cell cycle were conducted using immortalized or transformed cell lines. An inherent characteristic of transformed and immortalized cells is that normal cell cycle control mechanisms have been altered, and these alterations may change the ultimate consequence of HBx expression in a particular cell line. We conducted our studies with cultured, primary rat hepatocytes that were carefully monitored to ensure the maintenance of characteristics of normal, differentiated hepatocytes, the primary target of an HBV infection. Second, while some previous studies have analyzed the impact of HBx on the cell cycle were conducted using immortalized or transformed cell lines. An inherent characteristic of transformed and immortalized cells is that normal cell cycle control mechanisms have been altered, and these alterations may change the ultimate consequence of HBx expression in a particular cell line. We conducted our studies with cultured, primary rat hepatocytes that were carefully monitored to ensure the maintenance of characteristics of normal, differentiated hepatocytes, the primary target of an HBV infection. Second, while some previous studies have analyzed the impact of HBx on the cell cycle, none have specifically addressed the role of HBx in modulating cell proliferation pathways when HBx is expressed in the context of HBV replication; we addressed the specific impact of HBx on cell proliferation pathways when HBx was expressed in the context of HBV replication. Third, we have
linked HBx regulation of hepatocyte proliferation pathways to an identified fundamental activity of HBx; we have demonstrated that calcium signaling is required for HBx-induced modulation of hepatocyte proliferation. Finally, we have shown that HBV does not replicate efficiently in quiescent cells but instead requires an HBx-induced progression from G0 into G1 phase; HBV replication was inhibited when elevated p16 prevented progression into G1 phase, suggesting that HBV requires that cells are not in G0 but rather in G1 for efficient HBV replication. Overall, our studies demonstrate that HBx, when expressed in the absence of other HBV proteins and in the context of HBV replication, induces quiescent, normal hepatocytes to exit G0 but stall in the G1 phase of the cell cycle (Table 1 and Fig. 2 to 4); decreases the levels of p15 and p16, proteins that inhibit progression from G0 to G1 (Fig. 2B and 4A); increases the levels of the active G1-phase proteins p21, p27, cyclin D1, and cyclin E (Fig. 2C and 4B); and has no effect on the levels of proliferating cell nuclear antigen (PCNA) and cyclin A, S-phase proteins (Fig. 2D and 4C). We also showed that HBx causes an increase in the activity of the G1-phase kinase, cyclin-dependent kinase 4 (CDK4) (Fig. 3A and 4E), while inhibiting activation of CDK2, the late-G1/S-phase CDK (Fig. 3B and 4F). Finally, we demonstrated that HBx-induced modulation of hepatocyte proliferation pathways requires cytosolic calcium signaling (Fig. 5 and 6) and stimulates HBV replication (Fig. 7D).

Many DNA viruses that replicate in differentiated cells modulate cell proliferation pathways to enhance viral replication, and our studies suggest that a fundamental activity of HBx is to induce hepatocytes that are quiescent to enter the G1 phase of the cell cycle to create an environment that is more favorable for HBV replication (reviewed in references 39 and 41). Although some previous reports suggested that HBV replicates more efficiently in quiescent cells, these studies did not specifically address differences between the G0 and G1 phases of the cell cycle (23, 24). Our studies demonstrate that hepatocytes in G0 are not conducive to HBV replication and are consistent with previous reports in which HBV replication was shown to be higher in the G1 phase of the cell cycle (29, 46). We have demonstrated that HBV replication requires cell cycle entry; hepatocytes arrested in G0 phase by overexpression of p16 showed dramatically reduced levels of HBV replication. Because we noted that HBx prevented hepatocytes from progressing through S phase, consistent with previous studies of primary mouse hepatocytes (50), we did not specifically address whether HBV replication was altered in cells that are in S or G2 phase; previous studies suggested that HBV replication levels in HepG2.2.15 cells was decreased in S phase and elevated in G2 (29, 46). Whether HBV replication differs in the S or G2 phases in normal hepatocytes is the subject of our ongoing studies. Our demonstration that the phase of the cell cycle can affect HBV replication contradicts previous studies of

FIG. 6. HBx, when expressed in the context of HBV replication, requires cytosolic calcium signaling to modulate the levels and activities of cell cycle-regulatory proteins in primary rat hepatocytes. Hepatocytes were infected with AdHBV(HBx−) or AdHBV and treated with either 50 μM BAPTA-AM or a vehicle control. (A and B) Lysates were collected at 24 h postinfection; resolved via SDS-PAGE; and subjected to Western analysis for α-tubulin, p15, p16, HBcAg, p21, p27, and cyclin E. (C) Hepatocytes were collected at 24 h postinfection and analyzed for CDK4 activity by using a standard CDK4 kinase assay as described in Materials and Methods. Results shown are representative samples from at least 3 experiments performed in duplicate. *, statistically significant fold difference ± standard error between AdHBV(HBx−) and AdHBV as determined using Student’s t test (P = 0.05).
HBV replication in an HBV transgenic mouse model which suggested that HBV replicates equally well in resting and proliferating hepatocytes (23, 24). However, it is unclear whether HBV-transgenic mice, which contain a DNA copy of the HBV genome that is integrated into the host chromosome, fully recapitulate authentic HBV replication.

We did not specifically address why HBV requires cells that are in G0 to enter the G1 phase of the cell cycle to stimulate HBV replication while preventing cells from entering into S phase. One possible reason for HBx to induce hepatocytes to enter the G1 phase could be to increase the pool of available deoxyribonucleotide triphosphates (dNTPs). HBV requires host cell dNTPs to replicate its genome, and dNTPs are found at low levels in quiescent cells (69). The requirement for increasing the levels of dNTPs that are available for HBV replication might also be consistent with HBx-induced inhibition of progression from G0 to S phase, where the HBV replication machinery might compete with the cell replication machinery for available dNTPs. Alternatively, HBx might induce a transition from G0 to G1, to create an environment that is favorable for HBV replication, such as one with an increased pool of available dNTPs, while inhibiting progression into S phase to prevent activation of apoptotic pathways that sense unscheduled cellular DNA synthesis and cell replication. In this scenario, HBx-induced inhibition of progression into S phase would prevent activation of apoptotic pathways that could kill hepatocytes and impede HBV replication. An analysis of these various scenarios and their impact on HBV replication is the subject of our ongoing studies.

In addition to demonstrating that HBV replication in normal hepatocytes is stimulated by HBx-induced activation of G1 phase factors, we have linked HBx stimulation of cell proliferation pathways to its induction of calcium signaling. Previous studies showed that HBx elevates cytosolic calcium levels and activates Src kinases through a mechanism that is dependent on the mitochondrial permeability transition pore (MPTP) and that these HBx activities are required for HBV replication (4, 11, 12, 14, 43). The results outlined in the present study provide additional confirmation that a fundamental activity of HBx is modulation of cytosolic calcium signaling. In addition, these results are consistent with studies of primary mouse hepatocytes that linked an HBx-induced increase in the levels of p21 and p27 and G1-phase growth arrest to activation of MAPK signaling (50); previous studies indicated that HBx activates the MAPK signaling pathway by using a calcium-dependent mechanism involving the MPTP (4, 14, 33, 43). We are currently examining whether HBx-induced modulation of hepatocyte proliferation pathways is dependent on activities of the MPTP.

While the foci of our current studies have been to determine whether HBx regulates hepatocyte proliferation pathways, to link HBx-induced modulation of the cell cycle to a fundamental HBx activity, and to evaluate the consequence of HBx regulation of hepatocyte proliferation pathways for HBV replication, it is also quite likely that HBx-induced modulation of cell proliferation pathways may directly affect HBV-associated liver disease progression and the eventual development of HCC. Alterations in cell cycle proteins and their regulation are clearly involved in cancer progression and cellular transformation pathways (22), and HBx-induced changes in normal proliferation pathways in hepatocytes, especially its induction of cell cycle entry of normally quiescent hepatocytes, may facilitate HBV replication while ultimately proving detrimental to normal hepatocyte physiology. Consequently, understanding the molecular mechanisms that are associated with HBx-induced cell cycle progression and its effect on normal hepatocyte physiology may also help elucidate mechanisms that link chronic HBV infections to the development of liver cancer.

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