Hepatitis B Virus X Protein Inhibits Transforming Growth Factor-β-induced Apoptosis through the Activation of Phosphatidylinositol 3-Kinase Pathway*

Received for publication, April 27, 2000, and in revised form, May 31, 2000
Published, JBC Papers in Press, June 1, 2000, DOI 10.1074/jbc.M003578200

Wen-Ling Shih‡, Min-Liang Kuo‡‡, Shuang-En Chuang‡, Ann-Lii Cheng‡‡‡, and Shin-Lian Doong‡‡‡‡

From the ‡Graduate Institute of Microbiology, §Institute of Toxicology, and ¶Cancer Research Center, College of Medicine, National Taiwan University, the **National Health Research Institute, Taipei 11529, Taiwan and the ‡‡Department of Internal Medicine, National Taiwan University Hospital, Taipei 10019, Taiwan

Transforming growth factor-β (TGF-β) is a potent inducer of apoptosis in Hep 3B cells. This work investigated how hepatitis B virus X protein (HBx) affects TGF-β-induced apoptosis. Trypan blue exclusion and colony formation assays revealed that HBx increased the ID₅₀ toward TGF-β. In the presence of HBx, TGF-β-induced DNA laddering was decreased, indicating that HBx had the ability to block TGF-β-induced apoptosis. Furthermore, HBx did not alter the expression levels of type I and type II TGF-β receptors. HBx did not affect TGF-β-induced activation of promoter activities of the plasminogen activator inhibitor-1 (PAI-1) gene. These results indicate that HBx interferes with only a subset of TGF-β activity. In the presence of phosphatidylinositol (PI) 3-kinase inhibitors, wortmannin or LY294002, the HBx-mediated inhibitory effect on TGF-β-induced apoptosis was alleviated. In addition, the tyrosine phosphorylation levels of the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI 3-kinase) and PI 3-kinase activity were elevated in stable clones with HBx expression. Transactivation-deficient mutants of HBx lost their ability to inhibit TGF-β-induced apoptosis. Phosphorylation of the p85 subunit of PI 3-kinase and Akt, a downstream target of PI 3-kinase, was not observed in stable clones with transactivation-deficient HBx mutant’s expression. Thus, the anti-apoptotic effect of HBx against TGF-β can be mediated through the activation of the PI 3-kinase signaling pathway, and the transactivation function of HBx is required for its anti-apoptosis activity.

Hepatitis B virus X protein (HBx) has been demonstrated to function as a transcriptional transactivator of a variety of viral and cellular promoter/enhancer elements (1, 2). Although not binding directly to DNA, HBx can transactivate transcription through multiple cis-acting elements including AP-1, AP-2, ATF/CREB, NF-κB, C/EBP, and Egr-1 binding sites. However, the exact mechanism of transactivation still remains unsolved. Previous investigations have demonstrated that HBx interacts in the nucleus with components of the basal transcription machinery, including RPB5, a subunit of all three mammalian RNA polymerases, and several transcription factors (3–6). Thus, HBx may exert its effect by mimicking the cellular coactivator function. Another proposed mechanism for HBx activity involves the activation of signal transduction pathways such as the Ras/Raf/MEK/ERK, and MEKK-1/JNK cascades, leading to the induction of AP-1, NF-κB, and probably other transcription factors (7–12). HBx has been discovered to be distributed not only in the cytoplasm but also to some extent in the nucleus of transfected cells (9). Thus, HBx may have a dual function: one, related to its cytoplasmic localization, which can mediate the activation of signal transduction pathways, and another, a nuclear function, that may account for the interaction with transcription factors and components of the transcription apparatus to enhance the binding or activity of these proteins (9). In addition to its well known transcriptional transactivation ability through interaction with different cellular targets, HBx has been reported to affect DNA repair (13–17), cell cycle control (18, 19), and apoptosis (20–23). Therefore, the pleiotropic activities of HBx are potentially relevant to the development of hepatocellular carcinoma.

Transforming growth factor-β (TGF-β) is a potent inducer of apoptosis in hepatocytes and several hepatoma cell lines (24–26). TGF-β exerts its action through transmembrane serine/threonine kinase receptors. These receptors propagate the signal by phosphorylating the intracellular targets, Smads. Phosphorylated Smad2 or Smad3 can form a stable complex with Smad4, which then translocates to the nucleus to regulate the transcriptional response to TGF-β (27, 28). However, the mechanism(s) whereby TGF-β induces apoptosis is not fully characterized. Nevertheless, induction of oxidative stress (29), activation of caspase 3 (30), and inhibition of Rb expression (26) have been implicated in mediated TGF-β-induced apoptosis. In liver cells, insulin and insulin-like growth factor-1 (31), as well as interleukin-6 (32), all block TGF-β-induced apoptosis. Recent studies have revealed that phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream target, Akt, are responsible for the anti-apoptotic activity of these factors against TGF-β (32, 33).

To elucidate the correlation between the HBx gene and its response to apoptotic stimuli, the effect of HBx gene expression on TGF-β-induced apoptosis in the Hep 3B cell line was exami-

* This work was supported in part by the National Health Research Institute, Department of Health (DOH85-HR-529) and the National Science Council (NSC 89-2320-B-002-033) of Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Graduate Institute of Microbiology, College of Medicine, National Taiwan University, No.1, Section 1, Jen-Al Rd., Taipei, Taiwan 10019. Tel: 886-2-23123456 (ext. 8288); Fax: 886-2-23915293; E-mail: sldoong@ha.mc.ntu.edu.tw.

† The abbreviations used are: HBx, hepatitis B virus X; ERK, extracellular signal-regulated kinase; MEKK, mitogen-activated protein kinase (MEK/ERK) kinase; JNK, Jun terminal kinase; Jak, Janus kinase; STAT, signal transducers and activators of transcription; TGF-β, transforming growth factor-β; PI, phosphatidylinositol; GFP, green fluorescence protein; PAI-1, plasminogen activator inhibitor-1; PMT, polyclona middle T antigen.

Published, JBC Papers in Press, June 1, 2000, DOI 10.1074/jbc.M003578200
ined. Cells with constitutive or inducible expression of wild or mutant HBx were generated and tested. Transactivation-proficient HBx inhibited TGF-β-induced apoptosis. The PI 3-kinase/Akt signaling pathway was involved in the HBx-mediated anti-apoptotic effect.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Rabbit polyclonal antibodies against HBx expressed in *Escherichia coli* were employed for detection of HBx. Anti-HA (clone 12CA5) was purchased from Roche Molecular Biochemicals. Dr. R.-H. Chen of the National Taiwan University provided the anti-phosphotyrosine (clone 4G10) antibody was purchased from Upstate Biotechnology. Antibodies for Akt and phospho-Akt (Ser-473) were purchased from New England Biolabs. pGFpEmd-b was purchased from Packard. Finally, wortmannin and LY294002 were purchased from Sigma.

**Plasmid Construction**—pHBV48 (containing two tandem copies of the HBV genome, provided by Drs. H.-L. Wu and P.-J. Chen of the National Taiwan University Hospital) was employed as a template for polymerase chain reaction amplification of the HBx cDNA fragment. The 0.5-kilobase pair HBx cDNA fragment was inserted into NotI/blunt-ended pOP13/CAT (Stratagene) vector, generating pOP13pX. Two oligonucleotides (HA-U, CAT GTA CCC ATA CGA TGT TCC AGA and HA-L, CAT GGG AGC GTA ATC TGG AAC ATC GTA) were synthesized, annealed, and ligated into NcoI-cut pOP13pX, generating pOP13HApX. When expressed, pOP13HApX generated an N-terminal HA-tagged HBx. The tetracycline-regulated RevTet<sup>TM</sup>-system (CLONTECH) was applied for inducible expression of HBx and mutants. An 0.6-kilobase pair DNA fragment containing an HBx open reading frame was isolated after NcoI and BglII digestion of pHBV48, blunt-ended with Klenow fragment, and ligated with SalI/blunt-ended pRev-TRE (CLONTECH), generating pRT-X. After HindIII and Nael digestion of pGFpEmd-b, a 0.776-kilobase pair DNA fragment containing a GFP open reading frame was isolated, blunt-ended with Klenow fragment, and ligated with SalI/blunt-ended pRev-TRE (CLONTECH), generating pRT-GFP, pRT-HBx-GFP, pRT-HBx<sup>69</sup>GFP, pRT-HBx<sup>90–91</sup>GFP. When present in cells with rtTA expression, these plasmids generated a regulatable expression of the desired HBx-GFP fusion proteins. All of the constructs were confirmed by direct DNA sequencing.

**Cell Culture and Transfection**—Hep 3B cells were cultured in a minimum essential medium supplemented with Earle’s salt, 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and fungizone (1.25 μg/ml) at 37 °C in a 5% CO₂ incubator. The PT67 packaging cell line was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin G (50 units/ml), streptomycin (50 μg/ml), and fungizone (1.25 μg/ml) at 37 °C in a 5% CO₂ incubator. Transfection was performed by the calcium phosphate precipitation method according to the procedure described previously (34). For transient transfection, cells were harvested 48 h after transfection. For selection of stable clones, Hep 3B cells were cultured in the presence of 500 μg/ml G418 or 100 μg/ml hygromycin for 2 weeks depending upon the expression vector utilized. Resistant clones were selected, expanded, and assayed for expression of the transfected cDNA by Western blotting or immunoprecipitation.

**TGF-β-induced Cytotoxicity Assays**—2 x 10<sup>5</sup> Hep 3B cells were seeded onto a 35-mm tissue culture plate. 24 h after inoculation, cells were washed with phosphate-buffered saline and cultured in serum-free minimal essential medium for 48 h. TGF-β was then added to a culture medium at various concentrations. At 48 h after treatment, cells were collected by trypsinization and suspended in minimum essential medium supplemented with 10% fetal calf serum. Viable and nonviable cells were determined by direct counting using a hemocytometer in the presence of trypan blue. ID<sub>50</sub> was defined as the concentration of TGF-β able to reduce 50% of the viable cells. For the colony formation assay, after TGF-β treatment the Hep 3B cells were cultured in a complete medium for 2 additional weeks. The colonies were visualized by amido black staining.

**DNA Fragmentation Analysis**—Hep 3B cells, with or without TGF-β treatment, were collected, washed with phosphate-buffered saline, and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 1% Nonidet P-40). The supernatant was collected and incubated with RNase at a final concentration of 500 μg/ml for 1 h at 37 °C. Subsequently, proteinase K was added to a final concentration of 500 μg/ml. The mixtures were then incubated overnight at 55 °C. The DNA was extracted with phenol/chloroform, precipitated with ethanol, dissolved in TE<sub>8.0</sub> (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and subjected to 1.7% agarose-gel electrophoresis.

**Luciferase Assay**—p800luc, kindly provided by Dr. R.H. Chen, contained the luciferase expression unit driven by the TGF-β-responsive elements in the plasmidactivator-1 (PAI-1) promoter. p800luc and pRL<sup>kat</sup> were transfected into the designated cells. 24 h after transfection, the cells were serum-starved for 10 h and then treated with TGF-β (5 ng/ml) for an additional 12 h. Luciferase and β-galactosidase activities were quantified by the Luciferase Assay System and the β-galactosidase Enzyme Assay System (Promega), respectively. The luciferase activity was normalized to β-galactosidase activity to account for the transfection efficiency.

**Metabolic Labeling and Immunoprecipitation for HBx**—Cells were pre-incubated with methionine-, cysteine-, and serum-free Dulbecco’s modified Eagle’s medium for 1 h. Revidue Pro-mix<sup>1,25</sup>S in vitro cell labeling mix (Amersham Pharmacia Biotech) was added. Cells were then incubated at 37 °C for 2 h. After being washed twice with phenol-buffered saline, cells were lysed with lysis buffer (100 mM Tris-HCl, pH 7.5, 0.25% sodium deoxycholate, 2% SDS, 2% 2-mercaptoethanol, 2% Nonidet P-40, 10 mM dithiothreitol, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na<sub>VO</sub>₄, 10 μg/ml phenanthroline, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 16 μg/ml benzamidine HCl). Supernatants were then diluted 5 times with 50 μl Tris, pH 8.0. The HA-tagged HBx was precipitated from cell lysates by anti-HA or rabbit anti-HBx polyclonal antibodies followed by protein G-Sepharose (Amersham Pharmacia Biotech). The immunocomplexes were washed twice with NETN wash buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 0.25% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and subjected to SDS-polyacrylamide gel electrophoresis. The signals were visualized with a PhosphorImager (Molecular Dynamics).

**PI 3-Kinase Activity Assay**—PI 3-kinase activities were assayed according to a procedure described elsewhere (35). Briefly, 10<sup>6</sup> cells were washed twice with ice-cold phosphate-buffered saline and lysed with 1 ml of lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgC<sub>2</sub>₄, 1 mM CaC<sub>2</sub>₄, 1% Nonidet P-40, 10% glycerol, 1 mg/ml bovine serum albumin, 20 μg Tris, pH 8.0, 2 mM orthovanadate). Cell extracts were incubated with 1 μg of anti-phosphotyrosine antibody (Clone 4G10) overnight at 4 °C. The immunocomplex was precipitated with 50 μl of protein A-Sepharose for 1 h at 4 °C, washed three times with lysis buffer, twice with
LiCl buffer (0.5M LiCl, 100 mM Tris, pH 7.6), and twice with TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA). The immunocomplex was preincubated on ice for 10 min with 10 μM of 2 mg/ml PI (Sigma). Kinase reaction was performed by the addition of 40 μl of the reaction buffer (10 μCi of [γ-32P]ATP, 20 mM Hepes, pH 7.4, 20 μM ATP, 5 mM MgCl2) at room temperature for 15 min. The reaction was stopped by the addition of 100 μl of 1 M HCl and extracted with 200 μl of a 1:1 mixture of chloroform and methanol. The radiolabeled lipids were separated by thin-layer chromatography and visualized with a PhosphorImager.

RESULTS

HBx Protects Hep 3B Cells from TGF-β-induced Apoptosis—Hep 3B cells were transfected with pOP13HApX plasmid. In this plasmid, expression of wild type HBx protein fused to an N-terminal HA epitope was driven by a Rous sarcoma virus-long terminal repeat (RSV-LTR) promoter. G418-resistant colonies were selected, expanded, and verified for expression of HA-HBx fusion protein by immunoprecipitation. Fig. 1 indicates that a HA-HBx protein of the anticipated size (18 kDa) was detected in two representative clones, Hep 3B-H4 and Hep 3B-H13, respectively. Parental Hep 3B and a selected clone, Hep 3B-H11, with no detectable expression were included herein as a negative control. Trypan blue exclusion and colony formation assays were employed to assay cell viability after treatment with TGF-β. Fig. 2, A and B, illustrates that HBx expression resulted in a 3–4-fold elevation of ID₅₀ toward TGF-β. A DNA fragmentation assay was performed to verify that the increased viability of HA-HBx-expressing cells exposed to TGF-β is due to their resistance to the apoptotic effect of TGF-β. 10 h after the addition of 5 ng/ml TGF-β, a DNA laddering pattern (indicative of internucleosomal DNA cleavage) appeared, and it became apparent at 24 h in parental Hep 3B and Hep 3B-H11 cell lines (Fig. 2C). In contrast, two independent HBx-expressing cell lines (Hep 3B-H4 and Hep 3B-H13) showed little DNA fragmentation even at 24 h after exposure to the same amount of TGF-β. Therefore, our data indicated that HBx expression can block TGF-β-induced apoptosis.

HBx Affects Neither the Expression level of TGF-β Receptors nor the Transcription Activation Ability of TGF-β on the PAI-1 Gene—A previous study by Oshikawa et al. (36) illustrated that mink lung epithelial cells (MvLu) with HBx expression displayed a reduced growth inhibition response to TGF-β, which might be related in part to decreased expression of TGF-β receptors. Western blot analysis was performed to examine whether the resistance to TGF-β-induced apoptosis observed in Hep 3B cells with HBx expression is due to a cell surface loss of TGF-β receptors. Fig. 3A illustrates that parental Hep 3B as well as selected clones with or without HBx expression contained similar amounts of type I and II TGF-β receptors. Fig. 3B reveals the quantitative result after normalization with α-tubulin.

In addition to its ability to induce apoptosis, TGF-β possesses many biological activities, such as transcriptional activation or repression. The PAI-1 gene is highly induced by TGF-β and following TGF-β treatment. Cells were treated with TGF-β at the indicated concentrations. The viable cells were counted with a hemocytometer in the presence of trypan blue. The result was the average of three independent experiments, each performed in duplicate. B: clonogenic assay of Hep 3B, Hep 3B-H11, Hep 3B-H4, and Hep 3B-H13 following TGF-β treatment. Cells were treated with TGF-β at various concentrations for 48 h as indicated. Cells were then cultured in a complete medium for 2 additional weeks. The colonies were stained with amido black for direct counting. Data were the means of two independent experiments, each performed in duplicate. C: DNA fragmentation analysis. DNA was extracted from cells indicated at various times after treatment with (lower panel) or without (upper panel) 5 ng/ml TGF-β and subjected to agarose-gel electrophoresis.
frequently applied as an indicator of the effects of TGF-β on the production of the extracellular matrix (37). A reporter plasmid containing the TGF-β-responsive element in PAI-1 promoter was employed to evaluate the induction of the PAI-1 gene by TGF-β under the influence of HBx and to investigate whether HBx blocks other cellular responses to TGF-β. In Hep 3B cells, HBx affects neither the expression level of TGF-β receptors nor the transcriptional activation ability of TGF-β on the PAI-1 gene. A, levels of the type I and type II TGF-β receptors were determined by Western blot analysis. α-Tubulin was included as an internal control. B, after normalization with α-tubulin, relative amounts of type I and type II TGF-β receptors were compared with parental Hep 3B cells. C, PAI-1 promoter-driven luciferase reporter plasmid and β-galactosidase expression plasmid were co-transfected into the indicated cell lines. Luciferase activity was measured after cells were incubated with TGF-β for 12 h and expressed as a-fold induction relative to that from cells not treated with TGF-β. Data are the means ± S.D. of three independent experiments, each performed in duplicate.
HBx inhibits TGF-β-induced apoptosis.

**Point mutant HBx proteins were utilized to determine whether the transactivation function was required for the anti-apoptotic effect of HBx.** Pooled cells with doxycycline-inducible expression of green fluorescence protein (GFP), HBx, HBxGFP, HBx7GFP, HBx61GFP, HBx69GFP, and HBx90–91GFP were generated using the RevTet™ system (CLONTECH). Direct microscopic observation of green fluorescence (data not shown) or Western blot analysis confirmed expression of the desired products following TGF-β treatment in the absence (+) or presence (−) of 1 μg/ml doxycycline (Dox.). B, transactivation-deficient HBx mutants lost their ability to protect cells from TGF-β-induced apoptosis. After incubation with (lower panel) or without (upper panel) TGF-β (5 ng/ml) for 16 h, DNA was extracted and subjected to agarose-gel electrophoresis. C, viability of cells with expression of the indicated products following TGF-β treatment in the absence (−) or presence (+) of wortmannin using trypan blue exclusion assay.
The effects of HBx on cell death or apoptosis have been studied by several groups. p53-dependent apoptosis was prevented by microinjection of HBx into primary fibroblasts (18). Chirillo et al. (19) demonstrated that after DNA damage, HBx induced p53-dependent apoptosis in NIH3T3 cells transiently expressing HBx. In Chang liver cells, HBx failed to induce apoptosis; however, it did sensitize cells to apoptosis triggered by TNF-α (20). Upon the induction of HBx expression mediated by the Cre/loxP recombination system, liver cell apoptosis was observed independently of the p53 pathway (41). The liver cells derived from a transgenic mouse were more susceptible to diverse apoptosis insults, and this phenomenon was not dependent upon p53 (42). These seemingly contradictory results of HBx on apoptotic events might be attributable to the utilization of different cells and expression systems. However, these results suggest that HBx affects the apoptotic processes by multiple mechanisms, including the inactivation of the p53 functions, interference of DNA repair ability, or modulation of cellular signaling cascades. Our findings on the modulation of PI 3-kinase signaling by HBx in mediating its anti-apoptotic effect offered a new mechanism.

The molecular mechanism by which HBx activates PI 3-kinase is addressed hereafter. Because of its well known transactivation function through the increased expression of cytokines or cognate receptors, HBx might establish an autocrine or paracrine loop. HBx was reported to stimulate the expression of cytokines (e.g. interleukin-6 (43) and insulin-like growth factor-II (44)) as well as cytokine receptors (e.g. insulin-like growth factor-1 receptor and epidermal growth factor receptor). Interleukin-6 was demonstrated to inhibit apoptosis through the PI 3-kinase signaling pathway in hepatoma cells (32). The phosphorylated tyrosine residues, generated on receptors (e.g. epidermal growth factor receptor) or their associated substrate molecules (such as IRS-1/2 in signaling by insulin and insulin-like growth factor), form the docking sites for the Src homology-2 domains of p85. The interaction mediates the translocation of PI 3-kinase to the receptor tyrosine kinases and their substrate and assists in positioning p110, the catalytic subunit of PI 3-kinase, close to the membranes that contain the lipid substrates. Using Northern blot analysis or a ribonuclease protection assay, HBx did not alter the mRNA level of interleukin-6, TNF-α, or interferon-β (data not shown). The addition of media collected from the overnight culture of HBx-expressing cells did not protect Hep 3B cells from TGF-β-induced apoptosis (data not shown). Although not excluded, the hypothesis that the observed activation of PI 3-kinase by HBx might be due to the secondary results of the primary activation of cytokines or growth factors was not favored.

Rather, HBx might work through the modulation of signaling cascades to activate PI 3-kinase. Related investigations reported that HBx modulates several signaling cascades (7–12). Benn and Schneider (7) indicated that HBx activated Ras-GPT complex formation. Activation of Src family kinases was demonstrated to be indispensable for HBx-mediated activation of Ras (12). The activated Ras-GPT complex binds to p110, the catalytic subunit of PI 3-kinase, resulting in the activation of PI 3-kinase (47). The binding of the Src homology-3 domain of Src family kinases to a proline-rich region within the p85 of PI 3-kinase resulted in the activation of PI 3-kinase (48). Lately, HBx has been shown to interact with Jak1 and activate Jak-STAT signaling (49). Both Jak (50–52) and STAT (53) interact with p85 and activate PI 3-kinase signaling pathway. Therefore, through the activation of Ras, Src family kinases, or JAK-STAT, HBx might be able to achieve its effect on PI 3-kinase. In addition, HBx might directly activate PI 3-kinase. In a glutathione S-transferase pull-down assay, HBx was found

**DISCUSSION**

This study has demonstrated that HBx effectively suppresses TGF-β-induced apoptotic death of hepatoma cells. The HBx-mediated anti-apoptotic effect was not mediated through decreased expression of TGF-β receptors. Two specific inhibitors of PI 3-kinase, wortmannin and LY294002, blocked the anti-apoptotic effect of HBx, implying that HBx might affect the PI 3-kinase signaling pathway in mediating the effect. In cells expressing HBx, the PI 3-kinase activity and not its protein level was elevated. An increased phosphorylation of Akt at Ser-473 resulted. The anti-apoptotic mechanism of HBx was attributed, at least in part, to the activation of PI 3-kinase signaling cascades.
HBx Inhibits TGF-β-induced Apoptosis

...to be associated with p110 (data not shown). The contribution of this interaction in the observed elevation of PI 3-kinase activity requires further investigation.

It is noteworthy that wortmannin and LY294002 partially blocked the anti-apoptotic activity of HBx (Fig. 4). Although activation of the PI 3-kinase/Akt signaling pathway mediated the observed phenomenon, the interference of other molecules by HBx cannot be excluded. A recent investigation confirmed that HBx can inhibit caspase 3 activity (22). Chen and Chang (30) reported that caspase 3 was involved in TGF-β-induced apoptosis. However, the contribution of the inhibitory effect of HBx on caspase 3 in our system remains to be elucidated.

Polyoma middle T antigen (PMT) is identified as the tumorigenic component of the polyoma virus. PMT forms a complex with pp60c-src and PI 3-kinase, subsequently activating PI 3-kinase (54). Several studies have inferred that a PI 3-kinase signaling pathway is required for PMT-mediated tumorigenesis (35, 38). Our study clearly demonstrated the activation of PI 3-kinase signaling by HBx, subsequently triggering anti-apoptotic signaling. As with PMT, inhibition of apoptosis by HBx could disrupt the normal cellular surveillance mechanism for removing damaged cells, thereby providing a clonal selective advantage for hepatocytes expressing this integrated viral gene during the early stages of human liver carcinogenesis. Mutations that affected the transactivation activity of HBx inhibited its ability to activate PI 3-kinase/Akt signaling pathway and failed to block apoptosis. These observations indicate that transactivation and anti-apoptotic activity of HBx are linked. However, Gottlob et al. (55) have reported that transactivation activity is not required for the transforming activity of HBx in REV2 cells. Therefore, additional studies are required to further define whether PI 3-kinase activation and subsequently anti-apoptotic activity are a prerequisite for HBX-mediated transformation.

Acknowledgments—We thank Dr. Rey-Hwa Chen for valuable comments and Dr. H.-F. Yang-Yen for providing helpful instructions on the PI 3-kinase assay. We also acknowledge Mr. Ted Knoy for revision of the manuscript.

REFERENCES

1. Yen, T. S. B. (1996) J. Biomed. Sci. 3, 20–30
2. Murakami, S. (1999) Intervirology 42, 81–99
3. Cheong, J., Yi, M., Lin, Y., and Murakami, S. (1995) EMBO J. 14, 133–140
4. Qadri, I., Maguire, H. F., and Siddiqui, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1003–1007
5. Page, S. J., Yotsuyanagi, H., Iino, S., and Kurokawa, K. (1994) J. Virol. 68, 5408–5412
6. Fan, G., Ma, X., Kren, B. T., and Steer, C. J. (1996) Oncogene 12, 1909–1919
7. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 386, 465–471
8. Zhang, Y., and Derynck, R. (1999) Trends Cell Biol. 9, 274–279
9. Sanchez, A., Alvarez, A. M., Benito, M., and Fabregat, I. (1996) J. Biol. Chem. 271, 7146–7142
10. Chun, R.-H., and Chang, T.-Y. (1997) Cell Growth Differ. 8, 821–827
11. Tanaka, S., and Wands, J. R. (1996) Cancer Res. 56, 391–394
12. Chun, R.-H., Chang, M.-C., Su, Y.-H., Tsai, Y.-T., and Kuo, M.-L. (1999) J. Biol. Chem. 274, 23013–23019
13. Chun, R.-H., Su, Y.-H., Chuang, R. L. C., and Chang, T.-Y. (1998) Oncogene 17, 1959–1968
14. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
15. Oshikawa, O., Tamura, S., Kawata, S., and Matsuzawa, Y. (1996) Biophys. Biochem. Res. Commun. 222, 770–773
16. Keeton, M. R., Curriden, S. A., van Zonneveld, A. J., and Looktuffo, D. J. (1991) J. Biol. Chem. 266, 23048–23052
17. Dahl, J., Jureczak, A., Cheng, L. A., Baker, D. C., and Benitez, T. C. (1998) J. Virol. 72, 3221–3226
18. Kolik, G., Klippel, A., and Weber, M. J. (1997) Mol. Cell. Biol. 17, 1595–1606
19. Marte, B. M., and Downward, J. (1997) Trends Biochem. Sci. 22, 355–358
20. Shintani, Y., Yotsuyanagi, H., Moriya, K., Fujie, H., Tsutsumi, I., Kanegae, Y., Kimura, S., Saito, I., and Koike, K. (1999) J. Gen. Virol. 80, 3257–3265
21. Terradillos, O., Pollicino, T., Leocreuix, H., Tripodi, M., Gougeon, M. L., Tiollais, P., and Benito, M. L., Tiollais, P. (1998) Oncogene 17, 2115–2123
22. Lee, Y. H., and Yun, Y. (1998) Cancer Res. 58, 391–394
23. Tanaka, S., and Wands, J. R. (1996) Clin. Cancer Res. 2, 7721–7726
24. Park, U. S., Choi, I. Y., Yoon, S. K., Park, Y. M., and Lee, Y. I. (1998) Clin. Cancer Res. 4, 1711–1717
25. Lee, Y., Lee, S., Lee, Y., Bong, S. Y., Hyun, S. W., Yoo, Y. D., Kim, S. J., Kim, Y. W., and Poo, H. H. (1998) Oncogene 16, 2367–2380
26. Kim, S. O., Park, J. G., and Lee, Y. I. (1996) Cancer Res. 56, 3831–3836
27. Menzo, S., Clementi, M., Alfani, E., Bagarello, P., Lacovacci, S., Manzin, A., Dandri, M., Natoli, G., Levere, M., and Carloni, G. (1993) Virology 196, 878–882
28. Rodriguez-Viciana, P., Di Pietro, L., Fry, M., Waterfield, M. D., and Downward, J. (1994) Nature 370, 527–532
29. Pietenpol, C. M., Marc Hertz, W., and Cambier, J. C. (1994) Science 263, 1690–1612
30. Lee, Y. H., and Yun, Y. (1998) J. Biol. Chem. 273, 35510–35515
31. Sharpe, N., Dadi, H. K., and Roifman, C. M. (1995) Blood 86, 2077–2085
32. Oh, H., Fujio, Y., Kiniwin, K., Hirota, H., Matsu, H., Kishimoto, T., and Yamauchi-Takahara, K. (1998) J. Biol. Chem. 273, 9703–9710
33. Al-Shami, A., and Naccache, P. H. (1999) J. Biol. Chem. 274, 5333–5338
34. Pfeifer, L., Mullersman, J. E., Pfeifer, S. R., Murti, A., Shi, W., and Yang, C. H. (1997) Science 276, 1418–1420
35. Courtneidge, S. A., and Heber, A. (1987) Cell 50, 1031–1037
36. Gottlob, K., Pappu, S., Levere, M., and Grasemann, A. (1998) Cancer Res. 58, 3566–3570