Dual effects of hepatitis B virus X protein on the regulation of cell-cycle control depending on the status of cellular p53

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Introduction

Recent extensive studies demonstrated that hepatitis B virus (HBV) X protein (HBx) closely associated with chronic virus infection and hepatocellular carcinomas (HCCs). Especially, HBx induces liver cancer in some transgenic mice models (Kim et al., 1991) and is expressed in more than half of HBV-infected human livers and HCCs (Su & Schneider, 1996). In addition, HBx deregulates cell-cycle checkpoints (Benn & Schneider, 1994) and its overexpression causes uncontrolled cell proliferation (Yen et al., 1996). In many cases, failure to arrest cell proliferation generates cells with highly unstable genomes that could evolve into cancer cells (Jackson & Loeb, 1998; Martin-Caballero et al., 2001; Pruitt & Der, 2001).

Interestingly, it has been shown that the function of p53 can be suppressed by HBx via a protein–protein interaction(s) (Wang et al., 1994). In addition, transcription of p53 is strongly repressed by HBx (Lee & Rho, 2000). Moreover, HBx represses transcription of p21<sub>waf1/cip1</sub> via a p53-independent pathway, as demonstrated by Ahn et al. (2001). A variety of studies reported that p21<sub>waf1/cip1</sub> is an universal inhibitor of cyclin–CDK complexes and DNA replication and thus induces cell-cycle arrest at the G<sub>1</sub>–S checkpoint (Eapen et al., 2001; Galbiati et al., 2001; Pinski et al., 2001). Therefore, the altered expression of p21<sub>waf1/cip1</sub> by HBx might be important for the understanding of HBV-mediated HCC. However, it has been reported recently that HBx upregulates the expression of p21<sub>waf1/cip1</sub> and prolongs G<sub>1</sub>–S transition in human hepatoma cells (Han et al., 2000; Park et al., 2000), arguing that cells respond oppositely to HBx for the expression of p21<sub>waf1/cip1</sub> under different situations. Thus, we believe that the choice between cell-cycle arrest and progress through p21<sub>waf1/cip1</sub> mediated by HBx must be an important determinant to understand the mechanisms of hepatocellular carcinogenesis.

In this study, we have tried to investigate the effect of HBx on the activity of p21<sub>waf1/cip1</sub> in either the presence or the absence of its upstream regulator, p53, and to provide evidences that p21<sub>waf1/cip1</sub> activities mediated by HBx inhibit or stimulate cell-cycle progression depending on the status of cellular p53.

Methods

Plasmid construction. To construct the HBx-expressing plasmid, pCMV-3xHA1-HBx, the entire HBx sequence (nt 1374–1839, GenBank accession no. D23677) was inserted, in frame, downstream of three copies of the influenza virus haemagglutinin (HA) epitope [a nonapeptide sequence (YPYDVPDYA)] in pCMV-3xHA1 (Lee et al., 1998). The human p21<sub>waf1</sub> promoter construct, WWW-luc (p21<sub>P</sub>), was a gift from

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Bert Vogelstein (El-Deiry et al., 1993). p21^ΔA23 and other deletion mutants of the p21^waf1 promoter were described previously ( Datto et al., 1995 ).

### Transfection and luciferase assays.

HepG2, Hep3B and NIH3T3 cells were transfected by the calcium phosphate precipitation method, as described previously ( Ganem & Varmus, 1987 ). To control for the variation in transfection efficiency, 1 μg pCH110 (Pharmacia) containing the Escherichia coli lacZ gene under the control of the SV40 promoter was cotransfected as an internal control. At 48 h after transfection, the level of expression from the reporter gene (luciferase activity) was analysed and values obtained were normalized to the β-galactosidase activity measured in the corresponding cell extracts. Each experiment was repeated at least three times and the transfection efficiency, as determined by β-galactosidase-positive staining using the lacZ control plasmid, was at least 10% under optimal transfection conditions.

### Western blot analysis.

Transient transfections of HepG2, Hep3B and NIH3T3 cells for Western blot analysis were performed using the liposome reagent Fugene-6, according to the manufacturer’s protocol (Roche). Transfection efficiency was approximately 90%, as determined by β-galactosidase staining. Cells were lysed in buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1% SDS and 1% NP-40) supplemented with protease inhibitors. The protein concentration of the cell extracts was measured using the BSA Protein Assay kit (Bio-Rad). A 10 μg sample of each cell extract was separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Western blotting was performed with either anti-human p53 monoclonal antibody (Santa Cruz), anti-human p21 rabbit polyclonal IgG (Santa Cruz), anti-human actin monoclonal IgG (Santa Cruz) or anti-HA antibody (Roche) and subsequently detected using the chemiluminescent ECL kit (Amersham), as recommended by the manufacturer.

### RNA analysis.

Total RNA was prepared from cells using the guanidinium isothiocyanate procedure (Chomczynski & Sacchi, 1987). Total RNA (5 μg) was spotted onto a nitrocellulose membrane (Hybond-N; Amersham) and hybridized with an appropriate probe using the Detection Starter Kit II (Roche). For the preparation of probes, total cellular RNA was amplified by RT–PCR using appropriate pairs of primer and cDNA was labelled using the DIG labelling kit (Roche).

### Generation of stable cell lines and determination of cell growth rate.

NIH3T3 cells (2 × 10^5 cells per 60 mm diameter plate) transfected with 10 μg pCMV-3xHA1-HBx were selected and amplified to obtain stable cell lines, as described previously ( Chang et al., 1998 ). The expression level of HBx was checked by Western blot analysis. For the incubation under the appropriate conditions.

### Cell-cycle profiles were analysed using flow cytometry. Briefly, 2 × 10^6 cells were trypsinized, washed twice with PBS and fixed in 80% ethanol. Fixed cells were washed with PBS and resuspended in 50 μg/ml propidium iodide containing 125 U/ml RNase A. DNA contents were analysed by flow cytometry using cell-rrt software (Becton-Dickinson).

## Results

### Opposite effects of HBx on the expression of the p21^waf1/cip1 gene

To investigate the effects of HBx on early cell-cycle control in the presence or absence of p53, we employed a p53 reporter gene, WWW-luc, which contains the luciferase gene under the control of the 2.4 kb p21^waf1/cip1 promoter (El-Deiry et al., 1994) and cotransfected it with the HA-tagged HBx expression plasmid into a p53-positive hepatoma cell line, HepG2. Consistent with the previous report by Han et al. (2000), results from this experiment showed that HBx activated the promoter activity of p21^waf1/cip1 in p53-positive cells in a dose-dependent fashion (Fig. 1A). A comparable promoter construct, p21^ΔA23, which is driven by the truncation of the p21^waf1/cip1 promoter and thus is not responsive to p53 ( Datto et al., 1995 ), was not significantly affected by HBx (Fig. 1A, right panel), suggesting that HBx stimulates the transcription of p21^waf1/cip1 via a p53-dependent pathway. A similar experiment was performed in Hep3B cells, in which a functional p53 protein is absent ( Park et al., 2000 ). Interestingly, a completely opposite result was obtained, showing that HBx repressed the promoter activity of p21^waf1/cip1 in an HBx dose-dependent manner in these p53-negative cells (Fig. 1B, left panel). However, the repression of p21^ΔA23 reporter activity was largely maintained in p53-negative Hep3B cells (Fig. 1B, right panel). These results imply that, depending on the status of p53 in cells, HBx generates opposite effects on the transcription of p21^waf1/cip1 through completely different pathways. Using another luciferase construct, p21^ΔsmαA1, which contains only 61 bp proximal to the transcription initiation site ( Datto et al., 1995 ), was similarly repressed by HBx in Hep3B cells (data not shown), implying that the region of the promoter responsible for the repression by HBx in Hep3B cells might be in a 61 bp region near the transcription initiation site. Therefore, we are currently investigating the binding site responsible for the repression of p21^waf1/cip1 by HBx.

Based on the results above, we could suggest that HBx activates the p21^waf1/cip1 promoter in p53-positive cells in a p53-dependent manner, whereas it represses this promoter activity in p53-negative cells in a p53-independent manner. To confirm that the opposite effect of HBx on p21 promoter activity resulted from the difference in the status of p53, we tried to complement p53 in Hep3B cells by transient transfection with the p53-expressing plasmid and tested the effect of HBx in p53-expressing Hep3B cells. Increased p21^waf1/cip1 promoter activity by overexpression of p53 was more dramatic in the presence of HBx than in the absence of this viral protein (Fig. 1C), consistent with the ability of HBx to augment p21^waf1/cip1 promoter activity in p53-positive HepG2 cells but not in p53-negative Hep3B cells (Fig. 1A, B). Moreover, repression of p21^waf1/cip1 promoter activity by HBx in Hep3B cells was reversed in a p53 dose-dependent fashion (Fig. 1C). Thus, these results clearly indicate that the effect of HBx on the transcription of p21^waf1/cip1 is regulated by the status of endogenous p53.

To further demonstrate whether the activation of the p21^waf1/cip1 promoter in HepG2 cells is dependent on p53, a control luciferase construct (pT81-luc), containing a basic promoter element (TATA box), was employed. As expected,
Fig. 1. Effect of HBx on the transcription of p21\textsuperscript{waf1/cip1} in hepatic cell lines. (A) Activation of p21\textsuperscript{waf1/cip1} transcription by HBx in p53-positive cells. Increasing amounts of the HBx expression plasmid were cotransfected with 5 µg of the luciferase construct, in which transcription is driven by either a full-length form (p21P, left panel) or a truncated form (p21P\textsuperscript{Δ2.3}, right panel) of the p21\textsuperscript{waf1/cip1} promoter, into HepG2 cells. Subsequently, a luciferase assay was performed. The empty vector plasmid with three copies of the HA epitope was included as a control and to bring the concentration of effector protein up to 5 µg. (B) Repression of p21\textsuperscript{waf1/cip1} transcription by HBx in p53-negative cells. Hep3B cells were transfected as described in (A) and a luciferase assay was performed. (C) A sample of 5 µg of the p21\textsuperscript{waf1/cip1} luciferase plasmid was cotransfected with 0, 0.5, 1 or 2 µg of the p53-expressing plasmid into HepG2 cells in the presence or absence of HBx (5 µg). Luciferase activity was measured. (D) Each reporter plasmid was cotransfected with either an empty vector or a plasmid expressing the wild-type (wt) or a mutant form of p53 (p53 175H and 248Q, respectively) into cells in the presence or absence of HBx (5 µg). pCH110 (1 µg) was included in each transfection as an internal control and the relative activity of luciferase to β-galactosidase is presented. Relative luciferase activities from the p21\textsuperscript{waf1/cip1} promoter in the absence or presence of HBx are indicated by closed and open bars, respectively. Error bars indicate the SD obtained from three different experiments.

HBx did not affect basal reporter activity in both HepG2 (Fig. 1D) and Hep3B (data not shown) cells. However, in HepG2 cells, luciferase activity was increased approximately threefold by the presence of 13 copies of the p53-binding site in pTG13 (Lee et al., 1998) and was further activated about ninefold by HBx. In contrast, the p53-binding sites in pTG13 were not
affected by HBx in p53-negative Hep3B cells. However, the promoter activity of pTG13 in Hep3B cells was increased by the addition of the p53 expression plasmid and was further activated by HBx, whereas the addition of p53 naturally occurring mutants, such as p53-175H and 248Q, each of which has a fatal substitution in the DNA-binding domain at codons 175 (Arg → His) and 248 (Arg → Gln), respectively (Morgan et al., 2000), was not effective to stimulate p53 reporter gene activity mediated by HBx (Fig. 1D). These results confirm that HBx activates transcription of the p21waf1/cip1 gene through a p53-dependent pathway.

Since HBx has opposite effects on p21waf1/cip1 promoter activity depending on the status of p53, we examined the level of endogenous p21waf1/cip1 protein. Western blot analysis showed that the level of p21waf1/cip1 was increased by HBx in HepG2 cells (Fig. 2A, lanes 1 and 2) but was repressed in Hep3B cells (Fig. 2A, lanes 3 and 4). Furthermore, similarly to the promoter activity, the ability of HBx to induce the level of p21waf1/cip1 protein was restored when Hep3B cells were complemented with p53 (Fig. 2A, lanes 5 and 6), thus supporting the fact that the dual roles of HBx in the regulation of p21waf1/cip1 expression depend on the status of p53 in cells. Interestingly, the level of p53 was slightly increased in the presence of HBx in HepG2 cells (Fig. 2A, lanes 1 and 2). Apparently, it might not result from the effect of HBx on the promoter of p53 because a similar effect was also observed in which the p53 protein was introduced exogenously into Hep3B cells. To further elucidate whether the level of p21waf1/cip1 by HBx is influenced by transcription or translation, we isolated RNA from HepG2 cells and performed dot blot analysis showing that the level of p21waf1/cip1 RNA was clearly increased by the presence of HBx, whereas the level of p53 RNA was not significantly affected by HBx (Fig. 2B). These results imply that HBx activates transcription of p21waf1/cip1 not by the increased synthesis of p53 but by either the interaction or the modification of the p53 protein.

Interestingly, transcription of p21waf1/cip1 in NIH3T3 cells, which are p53-positive but maintain a low level of p53, was repressed about fourfold by HBx (Fig. 3A). We thus argued that this effect might be due to the very low expression level of p53 in NIH3T3 cells; a similar effect of HBx was observed in Hep3B cells when complemented with a low level of p53 (0.5 µg) (Fig. 1C). To demonstrate this possibility, we tried to increase the protein level of p53 in NIH3T3 cells by transient transfection of the p53-expressing plasmid and tested the effect of HBx on the p21 promoter. As the level of p53 increased, the repression fold of the p21waf1/cip1 promoter by HBx gradually decreased and was eventually reversed to activation when more than 1 µg of p53-expressing plasmid was added (Fig. 3A). A similar result was also obtained when the level of endogenous p53 was elevated by treatment with a DNA-damaging agent, cisplatin, which is widely used to induce the level of p53 (Fig. 3B). Western blot analysis showed that the level of p21waf1/cip1 protein in NIH3T3 cells was decreased by HBx but was increased when p53 was activated by treatment with cisplatin (Fig. 3C). The level of p53 in NIH3T3 cells seemed to be too low to show the stabilization effect by HBx but was clearly increased by HBx when the basal level was elevated by treatment with cisplatin.

### Opposite effects of HBx on cell-cycle progression

Because the tumour repressor protein p21waf1/cip1 is an universal inhibitor of cyclin–CDK complexes and DNA replication, it induces cell-cycle arrest at the G1–S checkpoint (Kaufmann et al., 2001; McDonald & El-Deiry, 2001); the altered expression of p21waf1/cip1 by HBx may effect cell growth. Thus, we prepared several NIH3T3 cell lines that

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**Fig. 2.** Effect of HBx on the expression of p53 and p21waf1/cip1. (A) Effect of HBx on the level of p53 and p21waf1/cip1. Either HepG2 (lanes 1 and 2) or Hep3B (lanes 3–6) cells were transfected with 5 µg of either an empty vector (lanes 1, 3 and 5) or an HBx-expression vector (lanes 2, 4 and 6). For lanes 5 and 6, 3 µg of the p53-expression plasmid was included in the transfection mixtures. Western blot analysis was performed using anti-p53, anti-p21waf1/cip1, anti-actin or anti-HA antibodies. Bound antibodies were visualized by chemiluminescence. (B) Effect of HBx on the level of p53 and p21waf1/cip1 RNA. HepG2 cells were transfected with 5 µg of either an empty vector (spot 1) or an HBx-expression vector (spot 2). A sample of 5 µg of DNase I-digested total cellular RNA was spotted onto a nitrocellulose membrane and hybridized with a DIG-labelled probe to detect RNA transcripts. As a control, the level of G3PDH RNA was measured.
Dual effects of HBx on cell-cycle control

Fig. 3. Effect of HBx on the expression of p21<sup>waf1/cip1</sup> in NIH3T3 cells. (A) The luciferase construct (p21P, 5 µg) was cotransfected with the p53-expressing plasmid at the indicated amount together with 5 µg of either an empty vector (closed bars) or an HBx-expression vector (open bars) into NIH3T3 cells and a luciferase assay was performed. (B) NIH3T3 cells were cotransfected with 5 µg of p21<sup>waf1/cip1</sup> luciferase construct (p21P) and 5 µg of either an empty vector or an HBx-expression vector for 36 h and then treated with cisplatin at the indicated concentration for an additional 12 h. (C) NIH3T3 cells were transfected with 5 µg of either an empty vector (lanes 1, 3 and 5) or an HBx-expression vector (lanes 2, 4 and 6) for 36 h and then treated with cisplatin at the indicated concentration. After an additional incubation for 12 h, total cell lysates were prepared and Western blot analysis was performed, as described in Fig. 2(A).

stably expressed HBx (NIH3T3/HBx); five different cell lines were selected and tested to show the differences in the growth rate. As expected, the growth rate of HBx-expressing cell lines, as presented by a representative one in Fig. 4(A, B), was approximately twofold faster compared with that of the parental NIH3T3 cells, indicating that the repression of p21<sup>waf1/cip1</sup> by HBx in NIH3T3 cells could be properly reflected by the stimulation of cell growth. When cells were treated with 5 µM cisplatin, both the parent NIH3T3 cells and the HBx-expressing cells progressively decreased in total cell number. However, the growth rate of HBx-expressing cells decreased more dramatically compared with that of the parent cells (Fig. 4A). Furthermore, the percentage difference in the total cell number between HBx-expressing cells and control cells increased as more cisplatin was added (Fig. 4B). From the data presented here, the rapid reduction in total cell number observed in the HBx-expressing cells could be explained by the effect of the increased levels of p21<sup>waf1/cip1</sup> in HBx-expressing cells to induce cell-cycle arrest at the G<sub>1</sub>–S checkpoint and/or to induce apoptosis caused by prolonged
Fig. 4. Effects of HBx on cell growth. (A) 5 × 10⁴ NIH3T3 cells (▲, △) or HBx-expressing stable cells (NIH3T3/HBx, □, ▪) were plated in 6-well plates. After 24 h, cells were treated with 5 µM cisplatin for the indicated period and the total cell number in each well was counted. (B) 1 × 10⁴ cells were treated with cisplatin at the indicated concentration for 24 h and the total cell number of the HBx-expressing cells was compared to that of the control cells. (C) Effect of HBx on cell-cycle progression. Cells were treated with 0, 5 and 25 µM cisplatin as indicated and cells were stained with propidium iodide and analysed by flow cytometry to determine the DNA content. The percentage difference in the size of the G₁, S and G₂/M cell population was determined by the comparison of HBx-expressing NIH3T3 cells with the parent cells.

Discussion

In this study, we demonstrated that the transcription level of p21⁰waft/cip⁰ could be determined by the balance between p53-dependent activation and p53-independent repression by HBx. Under this situation, p53 plays a key role in determining the final cellular events. When functional p53 is maintained over a certain level, the p53-dependent activation effect of p21⁰waft/cip⁰ by HBx might be dominant and thus overrides the repression effect by HBx. However, in the absence of functional p53, the p53-independent repression of p21⁰waft/cip⁰ mediated by HBx is involved in uncontrolled cell proliferation. The opposite effects of HBx on the transcription of p21⁰waft/cip⁰ depending on the status of p53 demonstrated in this study might be important to understand the progression of hepatic cell-cycle arrest. To further explore the effect of HBx on the progress of the cell cycle, we performed flow cytometry experiments on NIH3T3 cells in which the level of p21⁰waft/cip⁰ was increased by HBx when p53 was activated by cisplatin treatment. As shown in Fig. 4(C), in the presence of cisplatin, the proportion of cells in the G₁ phase clearly increased in HBx-expressing NIH3T3 cells compared to the parent cells. When cells were treated with cisplatin for a longer period or at a higher concentration, the G₁ proportion of HBx-expressing cells increased up to 80% compared to the parent cells in their percentage difference, whereas cells in other stages, S and G₂/M, decreased significantly (Fig. 1C). Therefore, the kinetics of the cell-cycle profile clearly demonstrates that HBx can effect cell growth at the G₁/S phase depending on the status of p53.
diseases in HBV-positive patients. Therefore, it is possible that induction of p21waf1/cip1 by HBx in the presence of p53 may cause prolonged arrest in G1, lead to apoptosis and thus contribute to the development of hepatocarcinoma during an early stage of virus infection. This hypothesis is consistent with the observation that the expression of both p53 and p21 is increased in cases of chronic severe viral hepatitis compared with those of chronic mild hepatitis (Papakyriakou et al., 2002).

Furthermore, a significant increase of apoptotic liver cells was found in severe chronic viral hepatitis cases compared to patients with mild steatohepatitis (Papakyriakou et al., 2002), suggesting that apoptotic cell death might be involved in the pathogenesis of hepatocellular damage of viral hepatitis. In the meantime, expression of HBx may enhance liver cell susceptibility to carcinogen-induced mutagenesis, potentially implying increased liver cancer risk.

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References


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