Published online April 23, 2004

2202–2213 *Nucleic Acids Research, 2004, Vol. 32, No. 7*
DOI: 10.1093/nar/gkh553

Natural variants of hepatitis B virus X protein have differential effects on the expression of cyclin-dependent kinase inhibitor p21 gene

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Received March 1, 2004; Revised and Accepted March 30, 2004

ABSTRACT

Despite the extensive studies on the roles of hepatitis B virus X protein (HBx), the effects of HBx on the important cellular processes such as cell growth, cell transformation and apoptosis remain controversial. Our previous study showed that the balance between p53-dependent activation and p53-independent repression by HBx determines the expression level of cyclin-dependent kinase inhibitor p21. In the present study, we further demonstrate that HBx natural variants have differential effects on p21 expression. The critical sites in HBx were identified as residues Ser-101 for activation and Met-130 for repression, respectively. The HBx variants with Ser-101 instead of Pro-101 stabilized p53 more efficiently, probably by protecting it from the MDM2-mediated degradation. On the other hand, the Met-130-containing HBx strongly repressed p21 expression by inhibiting Sp1 activity. Overall, the effect of HBx on p21 expression seems to be determined by the balance between the opposite activities. Depending on their potentials to regulate p21 expression, HBx variants showed different effects on the cell cycle progression, and eventually on the cell growth rate, implicating its biological significance. The present study may provide a clue to explaining the contradictory results related to cell growth regulation by HBx as well as to understanding the progression of hepatic diseases in HBV-positive patients.

INTRODUCTION

Cell cycle progression is driven by the sequential activation of cyclin-dependent kinases (CDKs), which are subject to regulation by positive (cyclins) and negative (CDK inhibitors) effectors (1). One such negative effector is a universal CDK inhibitor p21 (2). Through binding to cyclin/CDK complexes, p21 prevents CDK-dependent phosphorylation, and subsequent inactivation, of the retinoblastoma protein (Rb), which negatively regulates cell cycle progression (3,4). While p21 can be transcriptionally regulated by the p53 tumor repressor protein (5) and is thus believed to participate in the execution of p53 effects, its expression can be stimulated through several pathways triggered by a number of agents including tumor growth factor β (TGF-β), phorbol esters, okadic acid, butyrate, interleukin 6, interferon-γ, retinoic acid and vitamin D_3 (6–13).

Hepatitis B virus (HBV) X protein (HBx) has been the focus of much attention in recent years because it is strongly implicated in hepatocarcinogenesis. HBx augments HBV pregenome transcription and HBV replication. However, most HBx functions have been documented in terms of transcription, signaling pathways, genotoxic stress responses, protein degradation, cell cycle control and carcinogenesis (14). Several studies demonstrated that HBx regulates expression of p21 gene, which might play important roles in HBV-mediated hepatocarcinogenesis. For example, HBx down-regulates it by suppressing the function of p53 via a protein–protein interaction (15). In addition, HBx represses it via a p53-independent pathway, probably through modulation of the cellular transcription factor Sp1 activity, as demonstrated by Ahn *et al.* (16). The altered expression of p21 by HBx deregulates cell cycle checkpoints and thus causes uncontrolled cell proliferation (17). However, the exact opposite results, demonstrating that HBx upregulates the expression of p21 and prolongs $G_1 \rightarrow S$ transition in human hepatoma cells, were also reported (18,19). It is not understood how cells respond oppositely to the same protein for the expression of the important cell cycle regulator. The apparent difference might result from different experimental conditions. Especially, the cell status, such as cell density, nutritional condition and genetic background, could affect the response of cells to HBx. In addition, differences in the expression level or in the regulatory potential of HBx in each study also can be considered. Recently, we demonstrated that HBx has dual effects on the expression of p21, depending on the status of cellular p53 protein (20). According to the study, HBx activates transcription of p21 through stabilization of p53 protein whereas represses it via a p53-independent pathway. In this study, we further investigated whether p21 expression is modulated oppositely when different HBx variants are employed. To prove this hypothesis, we obtained several HBx clones from sera of Korean patients with either hepatitis or hepatocellular carcinoma (HCC) and compared their

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potentials to regulate the p21 expression. In addition, we tried to define the regions of HBx responsible for the dual effects and to provide possible mechanisms involved in the processes.

MATERIALS AND METHODS

HBx natural variants

HBx variants were obtained from HBV genomic DNA samples prepared from sera of Korean patients by PCR amplification. A pair of PCR primers, HBx-HA1 forward (5′-GCT CTC GAG TGC TGC CAA CTG GAT-3′) and HBx reverse (5′-AAC TCT AGA TGA TTA GGC AGA GGT-3′) was described previously (20). An initial denaturation at 94°C for 5 min was followed by 30 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 1 min, elongation at 72°C for 1 min and a final extension at 72°C for 5 min. PCR products cloned into pT7 Blue vector (Novagen) were sequenced using an automated DNA sequencer (model 373A). The DDBJ/EMBL/GenBank accession numbers of the HBx variants were AY308738, AY308742, AY311588, AY308743, AY308737, AY308741, AY308740 and AY308739 for hbx2, HBX22, HBX11, HBX26, hbx1, HBX6, HBX3 and hbx4, respectively.

Plasmid construction

To construct the HBx-expressing plasmid pCMV-3×HA1-HBx, the entire HBx sequence (nt 1374–1838) was inserted into *Xho*I and *Xba*I sites, in frame, downstream of three copies of the influenza virus hemagglutinin (HA) epitope [a nonapeptide sequence (YPYDVPDYA)] in pCMV-3×HA1 (21). For the construction of HBX3–hbx2 and hbx2–HBX3, the fragment either upstream or downstream of the *Hin*cII site (nt 1682–1687) in HBX3 and hbx2 was exchanged. For the construction of hbx2SM, hbx2PK and hbx2SK, the Pro-101, Met-130 and Pro-101/Met-130 of hbx2 were substituted with Ser-101, Lys-130 and Ser-101/Lys-130 residue by PCR-directed mutagenesis. Similarly, the Pro-101 and Met-130 of hbx2 were changed into Phe-101 and Leu-130 for hbx2FM and hbx2PL, respectively, whereas the Ser-101 and Lys-130 of HBX3 were changed into Phe-101 and Leu-130 for HBX3FK and HBX3SL, respectively.

Transfection and luciferase assay

The human cell lines HepG2 and Hep3B cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were seeded at 2×10^5 cells per 60 mm diameter plate and transfected the next day with a calcium phosphate–DNA precipitation method, as described previously (22). The human p21 promoter construct WWW-luc (p21P) was a gift from Bert Vogelstein (5). p21P Δ2.3 and other mutants of the p21 promoter were described previously (6). pG13-luc (21), MDM-luc (23) and p2×Sp1-luc (16) were also described previously. For the mammalian two-hybrid assay, 2 μg of pG4-MDM2 was transfected with 2 μg of either pCMV-VP16 or pCMV-p53/VP16 (21) in the presence or absence of 5 μg of pCMV-3×HA1-hbx2 plasmid into HepG2 cells. The Gal4 reporter G5E1b-luc has been described previously (21). To control for the variation in transfection efficiency, 1 μg of pCH110 (Pharmacia) containing the *Escherichia coli lac*Z 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 luciferase activity was analyzed and the values obtained were normalized to the β-galactosidase activity measured in the corresponding cell extracts. Each experiment was repeated at least three times.

Western blotting analysis

Cells were lysed in buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40] supplemented with protease inhibitors. Protein concentration of cell extracts was measured using the bovine serum albumin (BSA) protein assay kit (Bio-Rad). Ten micrograms of cell extracts were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Western blotting was performed with anti-rabbit p21 polyclonal IgG (Santa Cruz), anti-mouse p53 monoclonal antibody (Santa Cruz), anti-mouse HA monoclonal antibody (Roche), anti-mouse actin monoclonal IgG antibody (Santa Cruz) or anti-mouse MDM2 monoclonal antibody (Santa Cruz) and subsequently detected by chemiluminescent ECL kit (Amersham) as recommended by the manufacturer.

Co-immunoprecipitation

HepG2 cells were transfected with HBx-expressing plasmid as described above. After 48 h, cells were washed twice in PBS and harvested by scraping into cold lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 0.5 mM DTT) supplemented with protease inhibitors. The cell extract was centrifuged at 12 000 r.p.m. at 4°C for 15 min and the supernatant was incubated overnight at 4°C with anti-mouse p53 monoclonal antibody (Santa Cruz). Protein A/G agarose beads (Santa Cruz) were incubated with the lysate for 2 h with gentle shaking. The beads were collected by centrifugation, washed three times with the lysis buffer and resuspended in SDS loading buffer. The immunoprecipitates were eluted from the beads by incubation at 95°C for 5 min. The eluted proteins were separated by SDS–PAGE. Western blotting was subsequently performed with anti-mouse MDM2 antibody (Santa Cruz).

Generation of stable cell lines and determination of cell growth rate

HepG2 and Hep3B cells (2×10^5 cells per 60 mm diameter plate) transfected with HBx-expressing plasmid were selected and amplified to obtain stable cell lines as described previously (24). The expression of HBx was confirmed by western blot analysis. For the determination of cell growth rate, 5×10^4 cells were plated in six-well plates (Nunc) and the total cell number in each well was counted after incubation under the appropriate conditions.

FACS analysis

Cell cycle profile was analyzed 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 analyzed by flow cytometry using the Cell-FIT software (Becton-Dickinson Instruments).

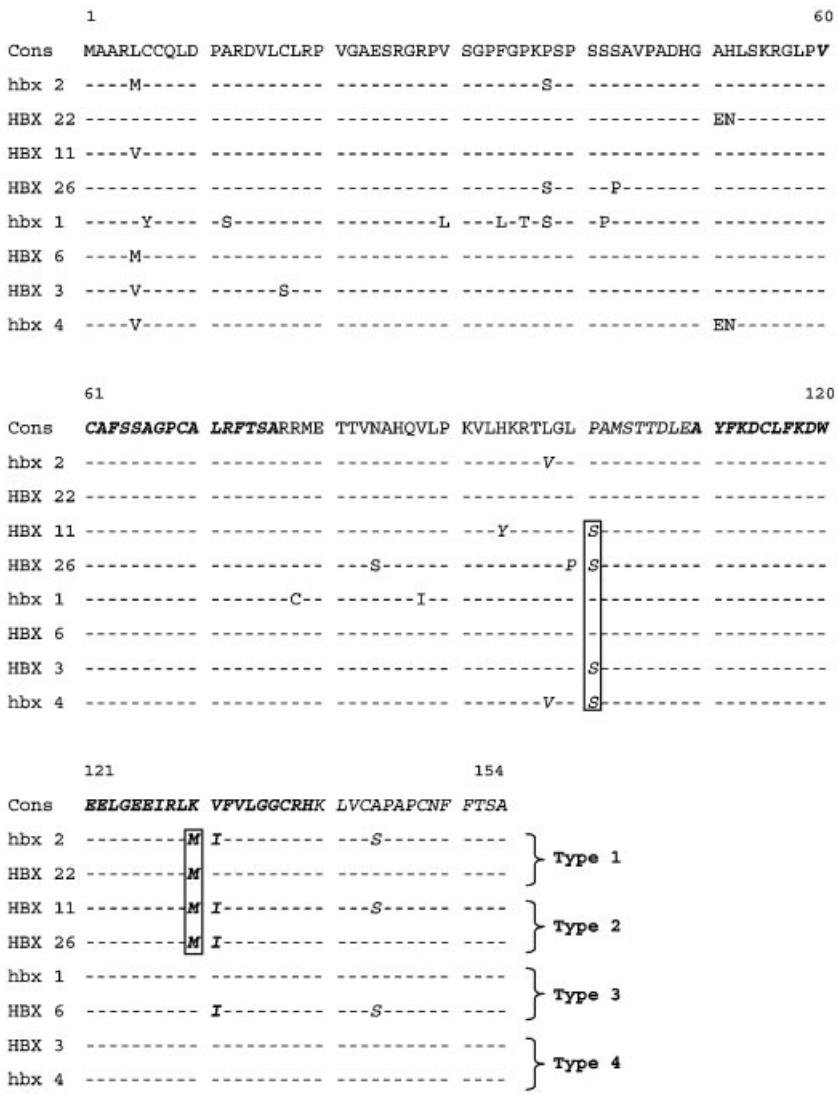

Figure 1. Alignment of the deduced amino acid sequences of HBx isolated from eight different Korean patients with either hepatitis (hbx) or HCC (HBX). Only the amino acids different from the consensus sequence of the isolates are indicated. The p53-binding domain and transactivation domain (25) of HBx are in italic and bold type, respectively. Both the Ser-101 and Met-130 residues important for the activation and repression of p21, respectively, are boxed. Each isolate was classified into one of four different types according to its p21 regulatory activities shown in Figure 2.

RESULTS

Four different types of HBx natural variants depending on the p21 regulatory potential

Twenty HBx clones were isolated from sera of Korean patients with hepatitis or HCC and the amino acid sequences of eight representatives are presented in Figure 1. According to the sequence comparison, a high level of amino acid sequence homology (94.2–98.1%) was observed throughout the entire region, including the transactivation domain (aa 60–76, 110–139) and the p53-binding domain (aa 101–154) (25). A specific sequence that can clearly distinguish HBx clones derived from hepatitis patients (hbx) and HCC patients (HBX) was not identified.

To investigate whether HBx variants have different effects on p21 expression, a luciferase assay using a reporter plasmid (p21P) that contains luciferase gene under the control of full-length p21 promoter (6) was performed. According to the result shown in Figure 2, the HBx variants shown in Figure 1 could be classified into four different types depending on their potentials to regulate p21 expression in the presence or absence of p53. Type 1 HBx (hbx2 and HBX22) has a strong p21 repression activity in p53-negative Hep3B (Fig. 2B) but exhibited almost no effects on p53-positive HepG2 cells (Fig. 2A). On the contrary, type 4 (HBX3 and hbx4) stimulated p21 expression strongly in HepG2 but showed almost no activity in Hep3B cells. Both type 2 (HBX11 and HBX26) and type 3 (hbx1 and HBX6) moderately stimulated p21 expression in HepG2 but only the former had a strong p21 repression activity in Hep3B cells. According to western blot analysis shown in Figure 2A and B, a similar pattern of p21 expression was observed in the HBx transfected cells. In addition, no significant differences in the expression level and protein size of HBx were observed in the transfected cells.

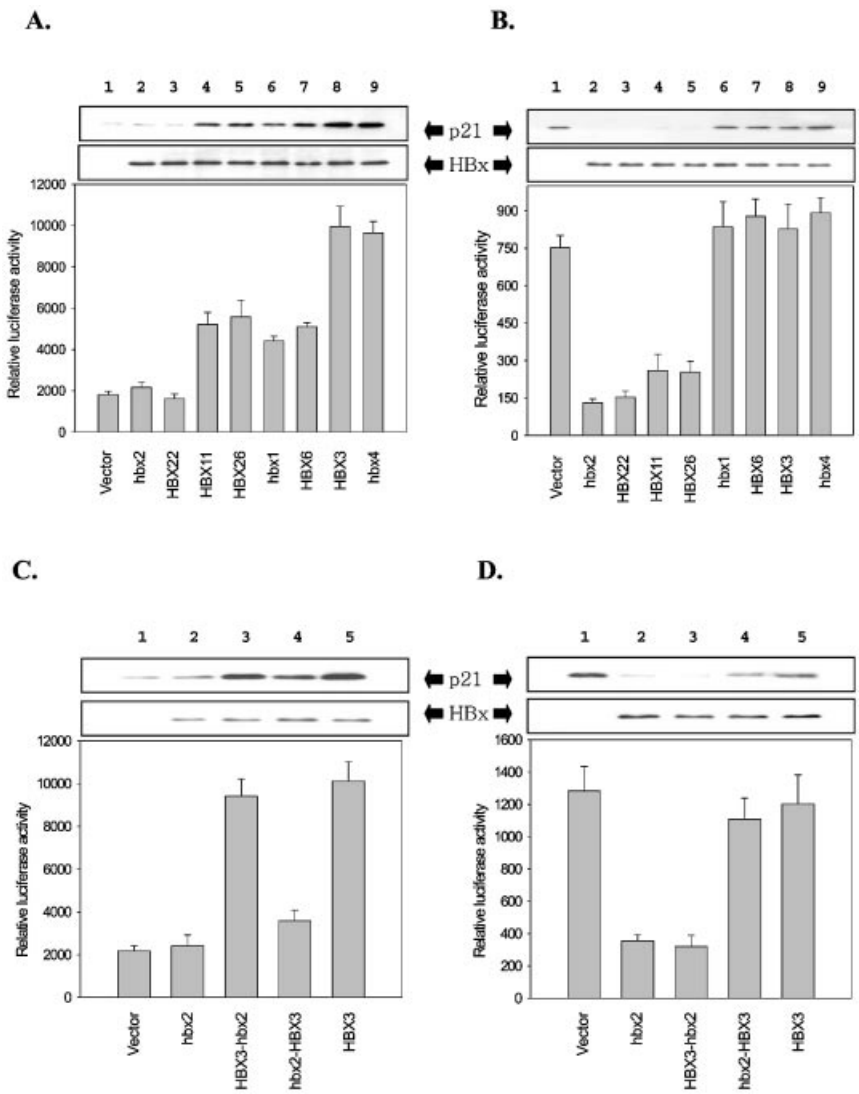

Figure 2. Dual effects of HBx on the transcription of p21 in hepatic cell lines. Five micrograms of the HBx expression plasmid was cotransfected with an equal amount of the luciferase construct (p21P), in which transcription is driven by a full-length form of the p21 promoter, into either HepG2 cells (**A** and **C**) or Hep3B cells (**B** and **D**). The empty vector plasmid with three copies of the HA epitope was included as a control. Error bars indicate standard deviations obtained from three different experiments. The protein level of p21 and HBx in each sample was determined by western blotting and is indicated in boxes above the corresponding values for luciferase activity.

Mapping of the regions in HBx responsible for the dual effects

HBx clones belonging to type 4 (HBX3 and hbx4) might have a specific motif(s) responsible for the activation of p21 promoter in HepG2 cells, which might be modified or absent in the type 1 HBx such as hbx2. To determine the sequence in HBX3 required for the activation of p21 in p53-positive cells, we made a recombinant HBX3–hbx2, which consists of the N-terminal two-thirds of HBX3 (aa 1–104) and the C-terminal one-third (aa 105–154) of hbx2. Similarly, another recombinant hbx2–HBX3 was also prepared. The potential of two recombinant HBx to activate p21 expression in HepG2 cells was tested along with their parent clones, hbx2 and HBX3. Interestingly, only HBX3–hbx2 stimulated p21 expression, up to a similar level obtained with HBX3, indicating that the N-terminal two-thirds of HBX3 is responsible for the activation of p21 (Fig. 2C). When tested in Hep3B cells, a comparable level of p21 repression was obtained with HBX3–hbx2 but not with hbx2–HBX3 (Fig. 2D), suggesting that the region responsible for the repression of p21 is located at the C-terminal one-third of hbx2. Therefore we concluded that the opposite effects of HBx on the p21 promoter were mediated through different regions of HBx protein, probably via different mechanisms.

The 101st and 130th amino acid residues in HBx determine the effects

We next examined the amino acid sequences of the HBx clones shown in Figure 1 to define the region responsible for the activation of p21. Interestingly, the serine residue at the 101st position (Ser-101) was conserved in type 4 but was substituted into a Pro residue in type 1 HBx. To demonstrate

whether Ser-101 is sufficient for the activation of p21, we artificially substituted Pro-101 of hbx2 (hbx2PM) into a Ser residue to generate hbx2SM. When introduced into HepG2, it significantly activated the expression level of p21 up to ~3-fold (Fig. 3A and B). In addition, the p21 stimulatory effect of HBX3 was significantly decreased when its Ser-101 residue was substituted into a Pro residue (data not shown). From the results shown above, the Ser-101 of HBx seems to be both sufficient and necessary for the strong activation of p21 in HepG2 cells. However, according to another substitution experiment shown in Figure 3E, HBX3FK in which Ser-101 of HBX3 was substituted into a Phe residue could activate the p21 expression to a similar level to that obtained with HBX3. In addition, an hbx2 recombinant containing a Phe residue instead of Pro-101 also activated the p21 expression. These results suggest that the Pro-101 in HBx might form a kink that alters the structure of HBx to a less effective form for the activation.

We also examined the C-terminal region (aa 106–154) of HBx clones in detail to define a critical sequence(s) responsible for the repression of p21. The Met-130 residue was conserved in members of types 1 and 2 but was substituted into a Lys residue in other HBx clones. To investigate whether Met-130 is important for the repression of p21, we constructed hbx2PK in which the Met-130 of hbx2 was substituted into a Lys residue. Surprisingly, such a substitution completely abrogated the potential of hbx2 to repress p21 promoter in Hep3B (Fig. 3C and D). In addition, after a substitution of Lys-130 into Met-130 was introduced, HBX3 also repressed the p21 promoter up to 8-fold in Hep3B cells (data not shown). To investigate whether Met-130 is absolutely required for the repression of p21 in Hep3B cells, we generated hbx2PL in which the Met-130 of hbx2 was substituted into a Leu residue. Hbx2PL could repress the p21 promoter in Hep3B to a similar level obtained with hbx2 (Fig. 3F), suggesting that Met-130 might be not essential for the effect. In addition, the expression of p21 was significantly repressed by HBX3SL in which Lys-130 of HBX3 was substituted into a Leu residue. Furthermore, HBX3SH in which the Lys-130 of HBX3 was substituted into another positively charged His residue did not show the repression effect (data not shown). Therefore the repression effect of HBx might be negatively regulated by the presence of a positively charged amino acid residue such as Lys at the 130th position.

Balance of the dual activities determines the effect of HBx

According to our previous report (20), the expression level of p21 in the presence of HBx is determined by the combination of the opposite effects. The different effects of HBx demonstrated in this study also can be explained by the balance of the opposite activities. Type 1 HBx (hbx2 and HBX22) and hbx2PM did not activate p21 expression in HepG2, possibly because the weak activation by the presence of Pro-101 was completely overridden by the repression effect conferred by Met-130. Type2 HBx (HBX11 and HBX26) and hbx2SM demonstrated a much lower level of p21 activation compared with type 4 HBx (HBX3 and hbx4) and hbx2SK (Fig. 2A and 3A) although they all contain the effective Ser residue at the 101st position. This might be due to the repression effect conferred by the Met-130 present in this group. This also indicates that the stimulatory Ser-101 is partially dominant over the inhibitory Met-130. The weak stimulatory Pro-101 residue present in members of type3 HBx (hbx1 and HBX6) and hbx2PK might be sufficient for the activation as their Lys residue at the 130th position was inactive for the repression. Finally, type 4 HBx and hbx2SK could highly activate p21 expression simply because the strong activation by the presence of Ser-101 was not interrupted by the repression effect.

It is possible to consider that type 1 HBx and hbx2PM resulted in strong p21 repression in Hep3B cells because they contain the weak stimulatory Pro-101 and the strong inhibitory Met-130. However, type 2 HBx and hbx2SM also produced a similar level of p21 repression in Hep3B cells (Figs 2B and 3C). In addition, substitution of the Pro-101 in hbx2PK into a Ser residue to generate hbx2SK hardly altered the effect in Hep3B cells. This can be explained if the activation effect of HBx required a functional p53 protein and thus could not be executed in p53-negative Hep3B cells. Although the downregulation of p21 by Met-130 is p53 independent, hbx2 and HBX22 clones that have Met-130 but not Ser-101 did not downregulate p21 in p53-positive cells. This indicates that Pro-101 is probably strong enough to overcome the repression effect conferred by Met-130. However, if p53 is not involved, hbx2 and HBX22 can downregulate p21 in both p53-positive and p53-negative cells as shown in Figure 3A and C.

p53-dependent activation of p21 expression by HBx

Based on the above results, we could suggest that HBx activates the p21 expression in p53-positive cells in a p53-dependent manner, whereas represses it in p53-negative cells via a p53-independent pathway. To confirm the p53-dependent activation of p21 expression by HBx, we employed p21P Δ2.3, which is driven by the truncation of p21 promoter and thus is not responsive to p53 (6). The truncated p21 promoter was not activated by hbx2SM and hbx2SK in HepG2 cells (Fig. 3A, right). Instead, the luciferase activity was significantly decreased by hbx2PM and hbx2SM, which probably resulted from the action of inhibitory Met-130 in the absence of the stimulatory effect. As expected, the repression was not observed with hbx2PK and hbx2SK that contain an inactive Lys residue at the 130th position.

To prove that the opposite effects of HBx on the p21 promoter activity in HepG2 and Hep3B 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 the p53-expressing Hep3B cells (Fig. 4A and B). The expression level of p21 in Hep3B cells was increased when p53 was exogenously complemented (Fig. 4A and B, lane 4). As expected, it was further activated by HBX3 but was slightly decreased by hbx2 (Fig. 4A and B, lanes 5 and 6), which is consistent with their ability to regulate p21 expression in p53-positive HepG2 cells. Addition of p53 naturally occurring mutants such as p53 175H, which has a fatal substitution in the DNA-binding domain at codon 175 (Arg to His) (26), was not effective to mediate the effect of HBx although its expression was similarly altered in the presence of HBx (Fig. 4A and B, lanes 7–9). These results confirm that the HBx activates transcription of the p21 gene through a p53-dependent pathway.

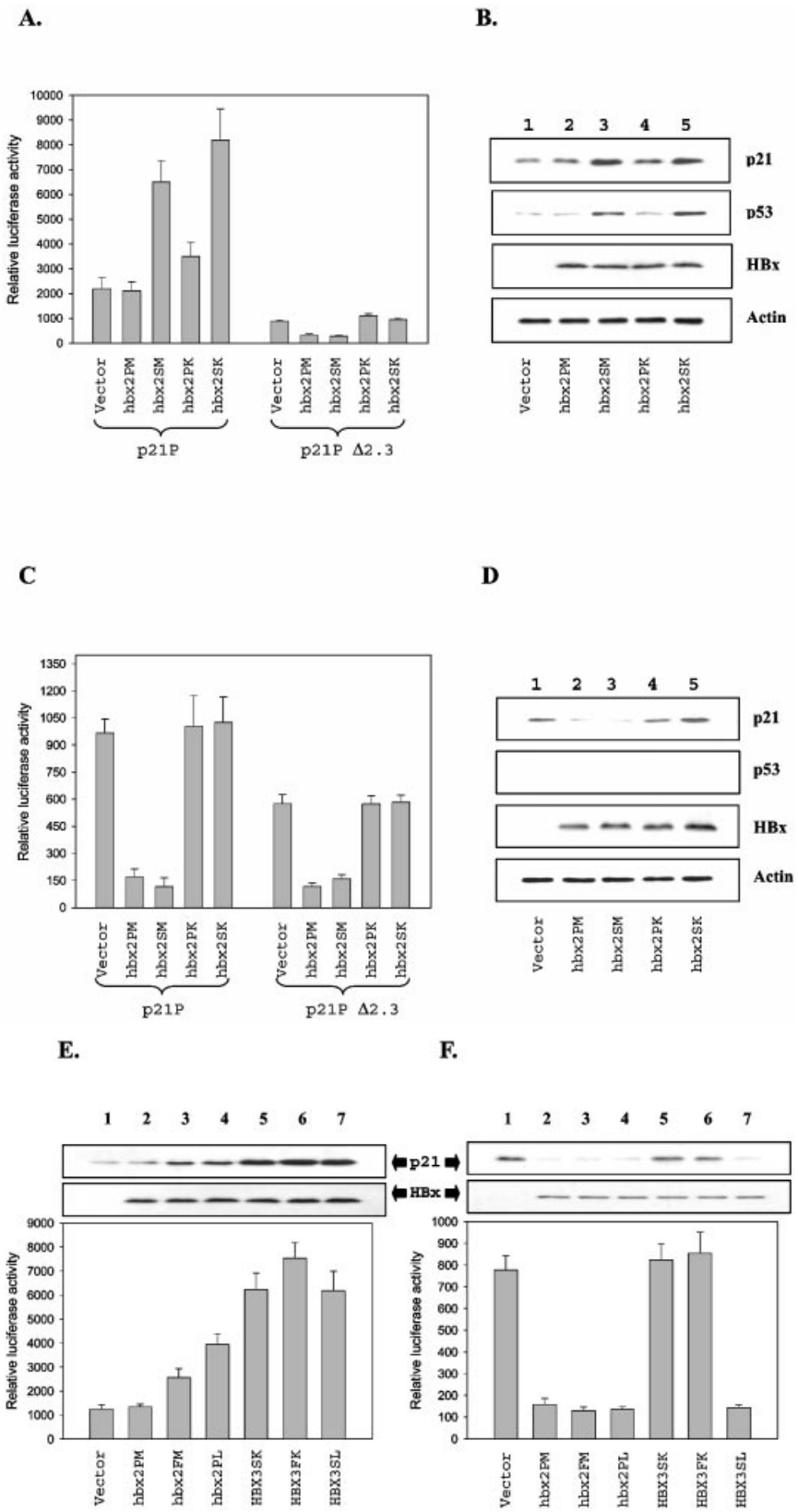

Figure 3. Determination of the HBx motifs responsible for the dual activities. Five micrograms each of HBx expression plasmid was cotransfected with an equal amount of the luciferase construct containing either a full-length form (p21P) (**A**, **C**, **E** and **F**) or a truncated form (p21P Δ2.3) (A and C) of the p21 promoter into either HepG2 cells (A and E) or Hep3B cells (C and F) and a luciferase assay was performed. The levels of p21, p53, HBx and actin in the HBx-transfected HepG2 (A) and Hep3B (C) cells were determined by western blotting and indicated in (**B**) and (**D**), respectively. The protein level of p21 and HBx in the transfected samples shown in (E) and (F) is indicated in boxes above the corresponding luciferase values.

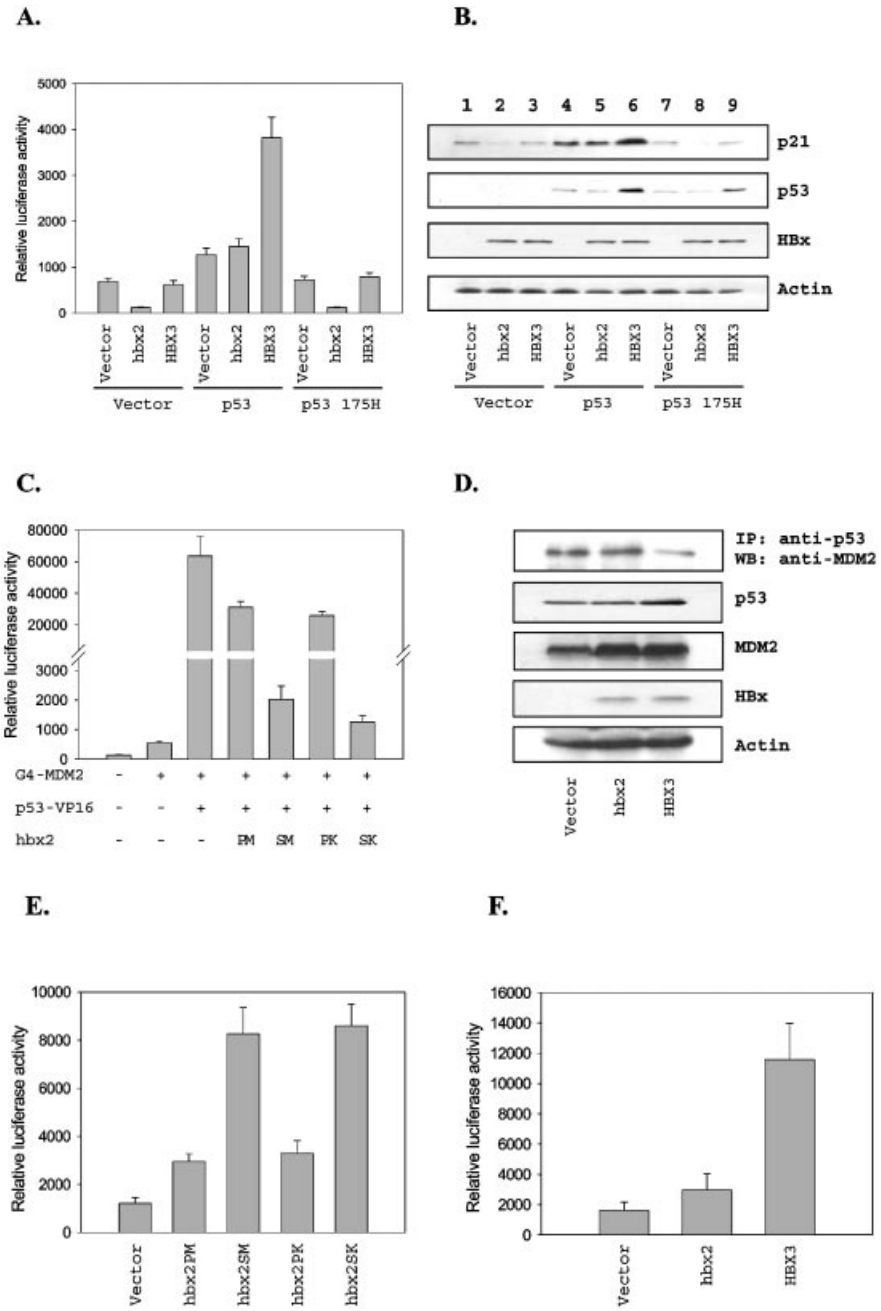

Figure 4. Mechanism of the p53-dependent p21 activation by HBx. (**A**) Five micrograms of p21 luciferase plasmid (p21P) was cotransfected with 2 μg of either wild type (p53) or mutant form (p53 175H) of p53-expressing plasmid into Hep3B cells in the presence or absence of HBx (5 μg) and luciferase activity was measured. (**B**) The protein level of p21, p53, HBx and actin in the transfected cells shown in Figure 4A was determined by western blotting. (**C**) The interaction between MDM2 and p53 in the absence or presence of HBx variants was measured by a mammalian two-hybrid assay. HepG2 cells were transfected with G4-MDM2 (2 μg) and p53-VP16 (2 μg) along with 5 μg of either an empty vector or an HBx-expressing construct. The luciferase activity from the Gal4 reporter (G5E1b-luc) was normalized to the β-galactosidase activity measured in the corresponding cell extract. (**D**) HepG2 cells were transfected with the indicated HBx expression plasmid (5 μg). At 48 h after transfection, cell extracts were subjected to immunoprecipitation using anti-p53 antibody (Santa Cruz) followed by western blotting using anti-MDM2 antibody (Santa Cruz). The levels of p53, MDM2, HBx and actin in the transfected cells were monitored by western blotting. (**E**) Five micrograms of pG13, which contains 13 copies of p53 binding site (21), was cotransfected with either an empty vector or plasmid expressing one of the hbx2 variants into HepG2 cells (5 μg each) and luciferase assay was performed. (**F**) p53-responsive promoter MDM-luc together with each HBx-expressing plasmid was transfected into HepG2 cells. The data presented reflect the average from three experiments of values normalized to the β-galactosidase activity from the internal control.

To elucidate the mechanism for the p53-dependent activation of p21 by HBx, we determined the expression level of p53 in the absence or presence of HBx. Surprisingly, the level of p53 protein was increased by hbx2SM and hbx2SK but not by hbx2PK and hbx2PM (Fig. 3B), suggesting that HBx stimulates the expression of p21 by increasing its upstream

regulator. Apparently, it might not result from the effect of HBx on the promoter of p53 because a similar effect was also observed in Hep3B cells in which the p53 protein was introduced exogenously (Fig. 4B). In general the action of p53 is negatively regulated by mouse double minute 2 (MDM2). MDM2 directly binds to the N-terminal domain (aa 1–43) of p53 and subsequently represses p53 transcriptional activity and mediates the degradation of p53 (27,28). Therefore we investigated whether HBx interferes with the binding of MDM2 and p53. In a mammalian two-hybrid assay using the bait G4-MDM2, strong stimulation of reporter gene activity occurred when VP16 activation domain-tagged wild-type p53 (p53-VP16) was included (Fig. 4C), indicating that MDM2 interacts with p53. Interestingly, the luciferase activity was decreased in the presence of HBx, suggesting that HBx may interfere with the interaction between MDM2 and p53. As predicted from their strong p53 stabilization effect, both hbx2SK and hbxSM more dramatically reduced the interaction between MDM2 and p53. Furthermore, according to the co-immunoprecipitation of p53 and MDM2 from the HBx-expressing HepG2 cells (Fig. 4D), the interaction between p53 and MDM2 was significantly reduced in the presence of HBX3, confirming that HBx with the Ser-101 residue stabilizes p53 by interfering with its interaction with MDM2.

Next, we investigated whether the increased p53 by the action of HBx still maintains its transactivation potential. The luciferase activity of pG13 (21), which contains 13 copies of p53 binding site, was significantly increased by the addition of HBx (Fig. 4E). Consistent with the effect on the p21 promoter, the luciferase activity increased more dramatically in the presence of the Ser-101-containing HBx (hbx2SM and hbx2SK). In addition, the stabilized p53 in the presence of HBx also could activate other p53 target promoters such as MDM2 (Fig. 4F) and BAX (data not shown). These results suggest that the stabilized p53 maintains its transcription regulatory activity.

p53-independent repression of p21 promoter by HBx

We previously reported that HBx represses p21 expression in a p53-independent manner by interfering with Sp1 activity (16). Consistently, some HBx isolates in this study could repress the p21 promoter in the absence of p53 (Figs 2 and 3). In addition, the truncated promoter that does not contain a p53-binding site was successfully repressed by hbx2PM and hbx2SM in Hep3B cells. Furthermore, they repressed the truncated promoter even in HepG2 cells in which p53 protein could not act on the promoter (Fig. 3A).

To investigate the mechanism by which hbx2 represses p21 in a p53-independent manner, we determined the regions of the p21 promoter responsible for the transcriptional repression by HBx. To this end, a series of progressive 5′ promoter deletion mutants of the p21 promoter were tested (Fig. 5A). These constructs were transfected into Hep3B cells with or without an HBx-expressing construct and the luciferase activity was measured. As expected, hbx2SK was not effective to repress the truncated p21 promoter. In contrast, hbx2PM could reduce the luciferase activity not only from the full-length p21 promoter but also from the 5′ truncated p21 promoter, including the minimal promoter construct p21P smaΔ1, which contains only 61 bp proximal to the transcriptional initiation site. Therefore the region responsible for the effect by HBx was determined to be a 61 bp region near the transcriptional initiation site.

To precisely define the regions of the p21 promoter necessary for repression by HBx, a panel of the point-mutated p21 promoter between bases –93 and –44 of p21P 93-S was examined (Fig. 5B). When tested in Hep3B cells, p21P 93-S mut1 was repressed by hbx2PM in a manner similar to that of the p21P 93-S. However, the repression fold by HBx was significantly decreased with p21P 93-S mut#2 and mut#3, and even almost completely disappeared with p21P 93-S mut#4 and mut#5 (Fig. 5B). All the constructs that were less responsive to HBx contained one or two Sp1 binding sites.

To determine if the Sp1 binding sites between –63 and –51 of the p21 promoter defined above are sufficient for the repression by HBx, this fragment was used in an attempt to confer HBx response to a heterologous promoter. A luciferase construct containing a consensus TATA box and initiator sequence, pGL2-basic, was nonresponsive to HBx (Fig. 5C). As expected from their ability to repress p21 promoter, both hbx2PM and hbx2SM repressed the Sp1 site whereas hbx2PK and hbx2SK did not (Fig. 5C). Therefore the repression of p21 by HBx variants might be determined by their ability to inhibit Sp1 activity, which is important for the proper expression of p21 (6).

Differential effects of HBx on the cell cycle progression

Because the tumor repressor protein p21 is a universal inhibitor of cyclin–CDK complexes and DNA replication, it induces cell cycle arrest at the $G_1 \rightarrow S$ checkpoint (29,30). To examine how the altered expression of p21 by HBx actually affects the cell cycle progression, we generated several HepG2 and Hep3B cell lines stably expressing hbx2 or HBX3 and compared their growth rate. As expected, the growth rate of HBX3-expressing HepG2 cell lines was more severely decreased compared with hbx2-expressing cell lines (Fig. 6B, left). Conversely, hbx2-expressing Hep3B cell lines, as presented by a representative one in Figure 6B (right), showed a higher growth rate compared with their parental Hep3B or HBX3-expressing cell lines. To demonstrate that the different growth rate resulted from the difference in $G_1 \rightarrow S$ progression in the cell cycle, we performed a flow cytometry experiment. As shown in Figure 6C, compared with the parent HepG2 cells, the proportion of G_1 phase more dramatically increased in HBX3-expressing HepG2 than in HepG2–hbx2 cells. On the other hand, the progression from G_1 to S phase might be more stimulated by HBx in Hep3B cells expressing hbx2 than in HBX3-expressing cells. Therefore it is possible to conclude that the differential effects of HBx variants on the p21 expression are properly reflected in the cell growth.

DISCUSSION

Several previous studies have demonstrated the interaction of p53 and HBx (31–33). The basic C-terminal domain of p53 and the C-terminal transactivation domain of HBx are involved in the process. As the HBx binding domain of p53 is also required for the formation of a stable p53 tetramer, it was suggested that the interaction inhibits both sequence-specific DNA binding and transcriptional activating properties of p53 (31,34). The present study, however, demonstrates completely different consequences of p53 and HBx

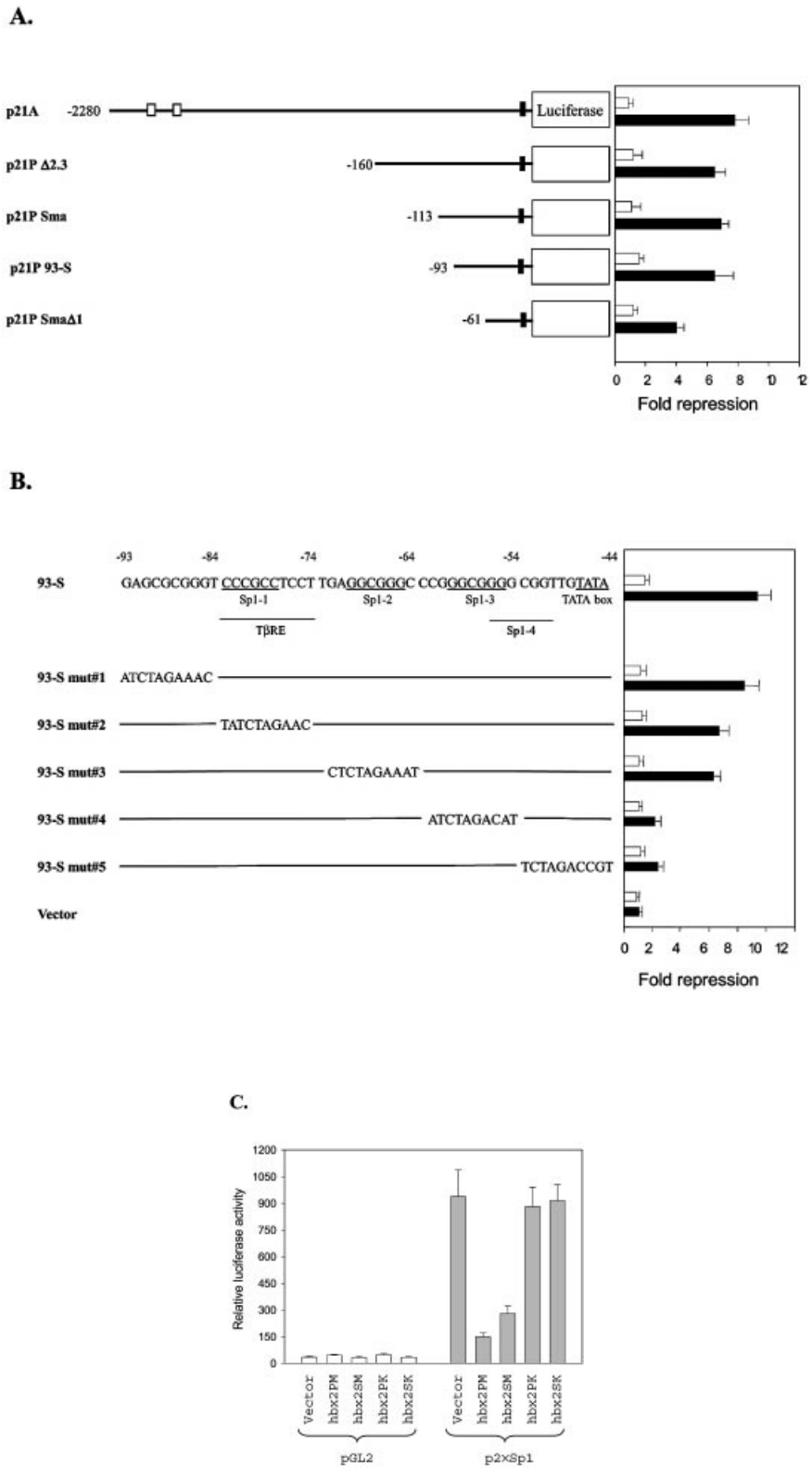

Figure 5. p53-independent repression of p21 by HBx. (**A**) Full-length and deletion p21 promoter constructs (6) were cotransfected with either hbx2PM-expressing plasmid (black bars) or hbx2SK-expressing plasmid (white bars) into Hep3B cells. Two p53 binding sites are indicated by open boxes. Repression fold was calculated by comparing the luciferase activity of HBx-expressing cells with the basal activity of the control. (**B**) 93-S mutant constructs, identical to the wild-type p21P 93-S sequence except the nucleotides shown for each mutant construct (6), were tested as above. The positions of transcription factor binding sites are underlined. (**C**) Sp1-mediated repression of p21 transcription by HBx. Five micrograms of p2×Sp1, in which the two Sp1 binding sites between –63 and –51 of the p21 promoter were inserted 5′ of the TATA box in pGL2 (16), was cotransfected with an equal amount of the indicated HBx plasmid into Hep3B cells and luciferase activity was measured.

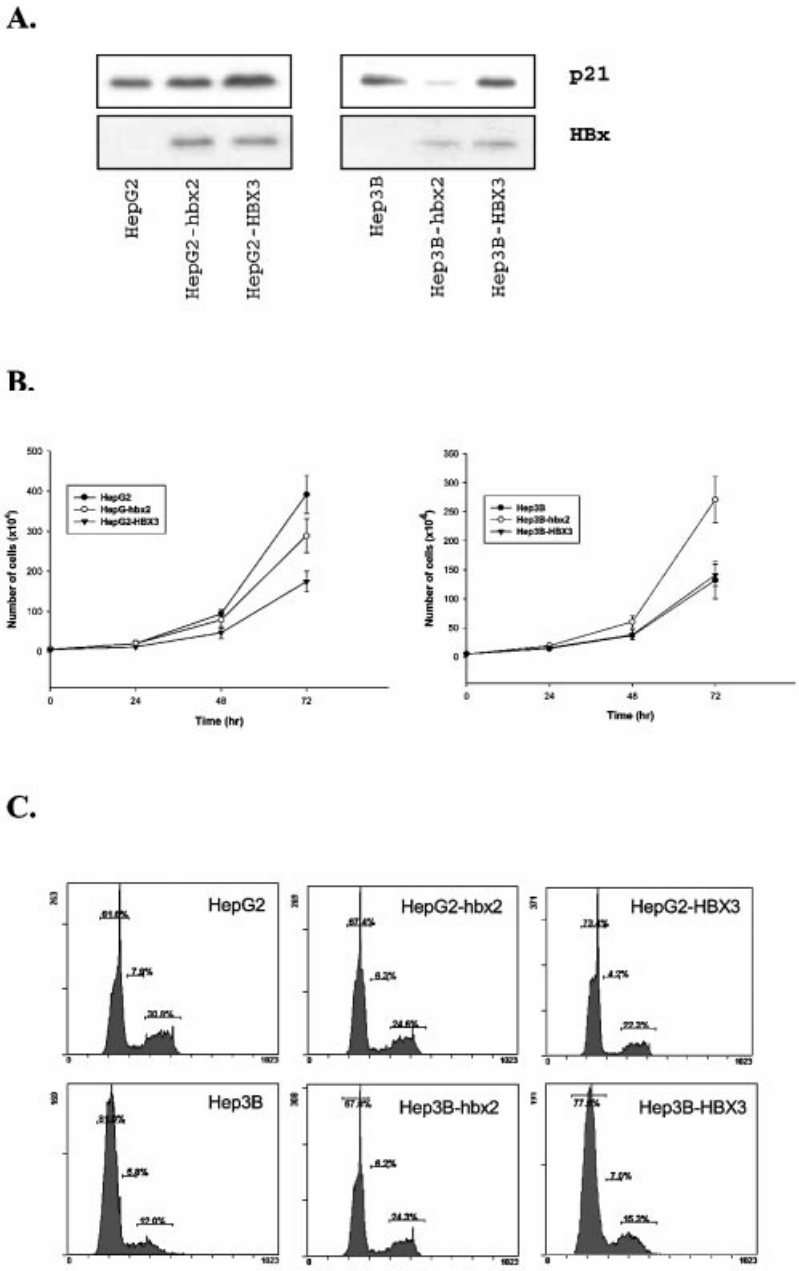

Figure 6. Effects of HBx on cell growth. (**A**) The expression level of p21 and HBx in stable cell lines (left, HepG2; right, Hep3B) was checked by western blotting analysis. (**B**) The growth rate of hbx2- and HBX3-expressing stable cell lines was compared with that of the parental cells. For the determination of cell growth rates, 5×10^4 cells were plated in six-well plates. The total cell number in each well was counted after the indicated period (left, HepG2; right, Hep3B). Error bars indicate standard deviations obtained from five different clones. (**C**) The cells were stained with propidium iodide and the cell cycle profile was analyzed by flow cytometry to determine the DNA content.

interaction. In the presence of some HBx natural variants, the activity of p53 for p21 transcription was upregulated, probably due to the increased protein level through stabilization. The potential of HBx to stabilize p53, thereby activating p21 expression, was decided by a specific amino acid residue (Ser-101) located at the p53-binding domain. It is unknown whether Ser-101 is involved in the interaction between p53 and HBx. According to our preliminary results, no clear differences in the interaction with p53 were observed among HBx variants (data not shown). In addition, the stabilized p53 by the action of HBx maintains its transactivation activity, which is not expected if p53 is stabilized via direct interactions with HBx. Instead, some HBx effectively interfered with the interaction between MDM2 and p53. Therefore HBx might stabilize p53 by inhibiting the action of MDM2 to direct p53 into a degradation pathway. MDM2 inactivation of p53 can be modulated by at least two mechanisms (35). Covalent modification of p53, for example by DNA damage-mediated phosphorylation, attenuates the interaction of MDM2 with p53. We are currently investigating the phosphorylation patterns of p53 in the presence of HBx. In addition, regulators of the p53–MDM2 interaction, such as the product of the

alternative reading frame (ARF) of the $p16^{INK4A}$ locus, can inactivate MDM2 and prevent destruction of p53. A role for HBx in modulating ARF function has not been demonstrated but is an intriguing possibility, especially as HBx is known to upregulate the level of Myc (36,37), which can activate ARF expression.

The present study may explain some contradictory reports about the effects of HBx on p53 and p21. HBx may increase p21 expression through p53 stabilization in p53-positive cells. On the other hand, some HBx variants can repress p21 through a p53-independent pathway when the p53 stabilization effect is weak or absent. Therefore the contradictory results reported so far might result from differences in the ability of HBx either to activate p53 or to repress p21 directly. However, with regard to some reports, it is true that the present hypothesis cannot explain the opposite results. Previously, HBx abolished p21 expression by inactivating p53 function by direct protein–protein interaction (15), and in some cases the upregulation of p21 is p53 independent (19). Therefore the mechanisms by which HBx regulates p21 expression seem to be much more complicated than expected. Some other variants, although not identified in this study, may lead to different kinds of protein–protein and/or protein–promoter interactions. In addition, these interactions can be influenced by other experimental conditions.

In accordance with their potential to regulate p21 expression, HBx variants could either stimulate or inhibit cell growth, indicating the biological significance of differential p21 regulation by HBx variants. Considering the multifunctional properties of HBx, however, HBx may alter cell growth rate in the absence of any change in p21 level, as demonstrated in this study with the decreased cell growth of HepG2–hbx2. Several studies have shown that expression of HBx can cause activation of both the mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways (38,39), which ultimately leads to the stimulation of cell cycle progression in established cell lines (40). On the contrary, expression of HBx can halt cell cycle progression, potentially via enhanced protein levels of p21 and p27 (41). It is not known whether the HBx variants described in this study have different effects on these intracellular signaling pathways.

The differential effects of HBx on the transcription of p21 demonstrated in this study are also important to understanding the progression of hepatic diseases in HBV-positive patients. It is possible that the increased p21 as a result of p53 stabilization by HBx causes the prolonged arrest in G_1, as demonstrated in this study. In this situation, the increased p53 may lead to the apoptotic cell death by modulating the level of proapoptotic Bax and antiapoptotic Bcl-2. Therefore the increased p53 and p21 in the presence of HBx may contribute to the development of hepatitis during an early stage of viral infection. This hypothesis is consistent with the observation that the expression of both p53 and p21 is increased in the cases of chronic severe viral hepatitis (42). During a long period of HBV replication in the liver of hepatitis patients, several mutations can be accumulated in the HBx coding region. Some HBx variants may lose their ability to stabilize p53 and thus cannot induce apoptotic cell death but maintain its potential to repress p21 in a p53-independent pathway. Actually, the double substitution K130M and V131I has been known to be more frequent in patients with cirrhosis and/or HCC than in patients with mild liver disease (43–47). Interestingly, according to the present study, most HBx isolates showing a strong p21 inhibitory activity have Met-130 and Ile-131 residues whereas Lys-130 and Val-131-containing HBx isolates did not showed the same activity at all, suggesting some biological significance of the present study. In addition, expression of HBx may enhance liver cell susceptibility to carcinogen-induced mutagenesis, potentially through downregulation of DNA excision repair by the HBx protein (48,49), thus conferring more chance on p53 to be inactivated. Actually, mutational inactivation of the p53 gene is very common (30–55%) in HCC (50). In this condition, the decreased p21 expression may induce rapid and uncontrolled cell proliferation and thus make it easy to accumulate more mutations on other oncogenes or tumor suppressor genes, ultimately leading to HCC.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Korea Health 21 R & D Project, Ministry of Health and Welfare, Republic of Korea (HMP-02-P-20203-0002). H.J.K. was also supported by the Brain Busan project in 2002.

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