Hepatitis B virus X protein activates proteasomal activator 28 gamma expression via upregulation of p53 levels to stimulate virus replication

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Abstract
Proteasomal activator gamma (PA28γ), frequently overexpressed in hepatocellular carcinoma, is believed to play important roles in tumourigenesis. However, the underlying mechanism of PA28γ overexpression and its possible roles in hepatitis B virus (HBV) replication are largely unknown. In the present study, we found that hepatitis B virus X protein (HBx) activates PA28γ expression by upregulating p53 levels in human hepatoma cells. The elevated PA28γ levels in turn repressed seven in absentia homologue 1 expression via downregulation of p53 levels, thereby inhibiting ubiquitin-dependent proteasomal degradation of HBx, which ultimately led to upregulation of HBx levels. The correlation among HBx, p53 and PA28γ was exactly reproduced in a 1.2-mer HBV replicon system, mimicking the natural course of HBV infection. In particular, knockdown of either p53 or PA28γ in HepG2 cells downregulated HBx levels and thereby inhibited HBV replication, whereas overexpression of p53 or PA28γ in Hep3B cells upregulated HBx levels, which stimulated HBV replication, indicating that p53 and PA28γ act as activators of HBV replication. In conclusion, HBx levels are upregulated via a positive feedback loop involving p53 and PA28γ to stimulate HBV propagation.

INTRODUCTION
Chronic infection with hepatitis B virus (HBV) is a leading cause of human hepatic diseases, including hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. As a member of the Hepadnaviridae family, HBV replicates via reverse transcription of a pregenomic RNA intermediate and encapsidates a partially double-stranded circular DNA genome of approximately 3200 base pairs [2, 3]. Of the four ORFs in the HBV genome, the shortest one encodes a 17 kDa regulatory protein, hepatitis B virus X protein (HBx). This multifunctional protein has been strongly implicated in HBV pathogenesis and HCC development because of its effects on diverse signalling pathways, transcriptional activation and dysregulation of immune responses, apoptosis and lipid metabolism [4, 5]. In addition, HBx’s role as a positive regulator of HBV replication has been demonstrated by several experimental systems, including chimeric mice with humanized livers [6], HBV transgenic mice [7], tail vein-injected mice [8], anti-HBx short hairpin RNA [9] and cell culture models [10]. For this role, HBx directly activates the four viral promoters to enhance HBV mRNA and pre-genomic RNA production from the covalently closed circular DNA template [11]. HBx may also indirectly contribute to virus replication by deregulating cellular signal transduction pathways, such as cytosolic calcium signalling [12] and the phosphatidylinositol 3-kinase/Akt pathway [13]. Despite our expanding knowledge of the roles of HBx in HBV pathogenesis and virus replication, the regulation of HBx levels during HBV replication remains poorly understood.

Proteasomes are protein complexes involved in the proteolytic degradation of unneeded or damaged proteins. Two distinct proteasomes differentially target proteins for degradation. The 26S proteasome, which is responsible for the degradation of the majority of proteins via a ubiquitin (Ub) and ATP-dependent pathway, is formed by association of the 20S catalytic core, composed of α and β subunits, with the 19S regulator [14, 15]. Alternatively, the 20S proteasome, which is responsible for Ub and ATP-independent degradation of specific target substrates, is generated by a
combination of one 20S catalytic core and one proteasomal activator 28 (PA28) member [14, 16]. Of the three PA28 family members, PA28 gamma (PA28γ; also called REG, 11Sγ, PSME3 or Ki antigen) is implicated in tumourigenesis, because it regulates cell proliferation and apoptosis, exerting its function predominantly through nuclear proteolysis [16]. For example, PA28γ contributes to p53 turnover by facilitating interactions between mouse double minute 2 (MDM2) and p53, thereby inhibiting DNA damage-induced apoptosis by limiting p53 accumulation [17, 18]. PA28γ also promotes the degradation of several cell cycle regulatory proteins, including p14, p16 and p21 [19, 20]. In addition, knockdown of PA28γ impaired the development of both hepatic steatosis and HCC in transgenic mice [21], indicating its role in the development of liver diseases. Consistently, PA28γ is frequently overexpressed in human cancers, including HCC [22]; however, the underlying mechanism is largely unknown.

Based on previous reports, it is possible to speculate a possible correlation among HBx, p53 and PA28γ. First, HBx upregulates p53 levels via activation of the ataxia telangiectasia mutated (ATM)-checkpoint kinase 2 (CHK2) pathway and enhances its transcriptional activity [23–25]. Second, p53 transcriptionally activates PA28γ expression through p53 response elements (p53REs) located within its promoter [26]. Third, HBx interacts with the host proteasome system to create an environment favourable for virus replication [27]. Therefore, it is challenging to investigate whether HBx activates PA28γ expression via upregulation of p53 levels and thereby dysregulates host proteasome activity to control its own protein level via a feedback system. In the present study, we first examined whether HBx transcriptionally activates PA28γ expression and upregulates its protein level via both ectopic expression and HBV infection systems. Second, we provide several lines of evidence supporting the view that HBx-mediated upregulation of p53 levels is responsible for the activation of PA28γ expression. Third, we investigated whether the activated PA28γ affects proteasomal degradation of HBx to modulate its protein level in a p53-dependent manner. Finally, we attempted to determine a possible role of this regulatory circuit in HBV replication.

RESULTS

HBx upregulates PA28γ levels via activation of p53 in human hepatoma cells

Initially, we investigated the effect of HBx on PA28γ levels in human hepatoma cell lines. Ectopic expression of HBx upregulated PA28γ levels in HepG2 and HuH7 cells in a dose-dependent manner (Fig. 1a). To prove the correlation between p53 and PA28γ, we attempted to knockdown p53 in HBx-expressing HepG2 cells, using a specific small hairpin RNA (shRNA). As a result, the potential of HBx to upregulate PA28γ levels almost completely disappeared in HepG2 cells (Fig. 1c). Unlike p53 shRNA, the scrambled (SC) shRNA used as a negative control did not affect p53 and PA28γ levels in HepG2 cells (Fig. 1c). In addition, ectopic p53 expression upregulated PA28γ levels in HepG2 and Hep3B cells in the absence of HBx (Fig. 1c), indicating that p53 upregulation itself is sufficient to upregulate PA28γ levels. Moreover, HBx also upregulated ectopically expressed p53 in Hep3B cells, resulting in further upregulation of PA28γ levels (Fig. 1c). Taken together, we conclude that HBx upregulates PA28γ levels via activation of p53 in human hepatoma cells.

HBx transcriptionally activates PA28γ expression via upregulation of p53 levels

Next, we investigated the mechanism by which HBx upregulates PA28γ levels in the presence of p53. It has been demonstrated that p53 transcriptionally activates the expression of PA28γ [26]. Consistently, data from both conventional and quantitative real-time RT-PCR showed that HBx dose-dependently upregulates PA28γ RNA levels in HepG2 but not in Hep3B cells (Fig. 2a). In addition, the PA28γ promoter activity was dose-dependently upregulated by HBx in HepG2 cells, but not in Hep3B cells (Fig. 2c). In contrast, the reporter activity from the PA28γmp53RE-luc construct, in which p53REs essential for the p53-mediated activation of PA28γ transcription [26] were ablated by point mutations (Fig. 2b), was minimally affected by HBx (Fig. 2c). Moreover, p53 knockdown almost completely abolished the potential of HBx to activate the PA28γ promoter in HepG2 cells, whereas ectopic p53 expression successfully restored HBx activation in Hep3B cells (Fig. 2d). In addition, ectopic p53 expression in the absence of HBx elevated the PA28γ promoter activity in Hep3B cells (Fig. 2d). Lastly, data from the chromatin immunoprecipitation (ChIP) assay showed that binding of p53 to the PA28γ promoter could be enhanced by HBx in a dose-dependent manner (Fig. 2e). On the basis of these observations, we conclude that HBx transcriptionally activates PA28γ expression via upregulation of p53 levels in human hepatoma cells.

PA28γ protects HBx from Ub-dependent and Ub-independent proteasomal degradation

Interestingly, the data from Fig. 1(a) showed a close correlation among p53, PA28γ and HBx in HepG2 cells. In addition, HBx levels decreased as p53 knockdown downregulated PA28γ levels in HepG2 cells, whereas HBx levels increased when ectopic p53 expression upregulated PA28γ levels in Hep3B cells (Fig. 1c). To confirm the correlation between p53 and HBx, the half-life of HBx in the presence or absence of p53 was compared. For this purpose, we
treated HepG2-HBx and Hep3B-HBx cells with cycloheximide (CHX) to block further protein synthesis, while chasing HBx and $\gamma$-tubulin levels in these cells. As shown in Fig. 3(a), the half-life of HBx in HepG2 cells was about two-fold longer (approximately 30 min) compared to that in Hep3B cells (approximately 15 min). Accordingly, the protein level of HBx in HepG2 cells was higher than that in Hep3B cells (Fig. 3b).

Moreover, treatment with a universal proteasomal inhibitor, MG132, upregulated HBx levels in both cell lines, while the effect was more dramatic in Hep3B cells (Fig. 3b). As a result, similar levels of HBx were detected in HepG2 and Hep3B cells in the presence of MG132. Therefore, HBx appeared to be less susceptible to proteasomal degradation, and thus more stable, in the presence of p53, suggesting that p53 is a positive regulator of HBx.

We then investigated the possible role of PA28g in the regulation of HBx. Both seven in absentia homologue 1 (Siah-1), an E3 Ub ligase of HBx and PA28g are transcriptional targets of p53 [26, 30], whereas PA28g is known to downregulate p53 levels via activation of MDM2 [18]. Consistently, ectopic PA28g expression downregulated p53 and Siah-1 levels both in the presence and absence of HBx, resulting in upregulation of HBx levels in HepG2 cells (Fig. 3c). In addition, PA28g knockdown upregulated both p53 and Siah-1 levels in HepG2 cells in the presence and absence of HBx (Fig. 3c, d). Accordingly, PA28g knockdown increased the ubiquitinated forms of HBx (Fig. 3g), resulting in downregulation of intact HBx in HepG2 cells (Fig. 3d, g).
Consistently, PA28γ knockdown in the nucleus almost completely removed the HBx signal in the cytoplasm of HepG2-HBx cells (Fig. 3e). These results suggested that PA28γ inhibits Siah-1 expression by downregulating p53 levels, thereby protecting HBx from Ub-dependent proteasomal degradation. Interestingly, however, both PA28γ and HBx levels were downregulated under the condition that p53 knockdown downregulated Siah-1 levels [24] and thereby decreased the ubiquitinated forms of HBx in HepG2 cells (Figs 1c and 3g). Moreover, both PA28γ and HBx levels were upregulated under the condition that ectopic p53 upregulated Siah-1 levels [24] and thereby increased the ubiquitinated forms of HBx in HepG2 cells (Figs 1c and 3g). The Siah-1-independent upregulation of HBx levels by PA28γ was also observed when PA28γ was overexpressed in Hep3B cells, which resulted in upregulation of HBx levels without affecting Siah-1 levels and HBx ubiquitination (Fig. 3f, g). Similarly, PA28γ knockdown downregulated HBx levels in Hep3B cells without affecting Siah-1 levels (Fig. 3f). Taken together, we conclude that PA28γ upregulates HBx levels by protecting it from Ub-dependent and Ub-independent proteasomal degradation.

**HBx activates PA28γ expression by upregulating p53 levels during HBV infection**

We then investigated whether HBx activates PA28γ expression during HBV infection. For this purpose, we employed a 1.2-mer HBV replicon system, which mimics the natural course of HBV infection [31]. Transfection with an HBV replicon, 1.2-mer WT, but not with its HBx-null counterpart, 1.2-mer HBx-null, upregulated both p53 and PA28γ levels in HepG2 cells (Fig. 4a). In addition, infection of WT HBV but not HBx-null mutant in HepG2 cells expressing sodium taurocholate cotransporting polypeptide (NTCP) as an HBV entry receptor [32] also upregulated both p53 and PA28γ levels (Fig. 4b). The HBV replication in these cells
**Fig. 3.** HBx escapes from proteasomal degradation by upregulating PA28γ levels. (a) HepG2-HBx and Hep3B-HBx cells were transfected with the indicated amount of p53 shRNA plasmid and p53 expression plasmid, respectively, for 24 h and then treated with 50 µg ml⁻¹ of CHX for the indicated time, followed by Western blotting. (b) HepG2 and Hep3B cells were transfected with the HBx expression plasmid for 48 h. Cells were either mock-treated or treated with 10 µM MG132 for 4 h before harvesting. The values indicate the level of expression.
was confirmed by the detection of viral proteins, including HBx, HBV core antigen (HBc) and HBV surface antigen (HBs) (Fig. 4a, b). IFA also detected a stronger PA28γ signal in HepG2 cells in which HBx was expressed from 1.2-mer WT (Fig. 4c). In contrast, none of these effects were observed in Hep3B cells (Fig. 4a, c). In addition, p53 knockdown in HepG2 cells transfected with 1.2-mer WT downregulated PA28γ and HBx levels, whereas ectopic p53 expression in Hep3B cells transfected with 1.2-mer WT upregulated their levels in a dose-dependent manner (Fig. 4d). These results were consistent with the data from HBx overexpression (Fig. 1). These results suggested that HBx activates PA28γ expression by upregulating p53 levels, thereby elevating its own protein levels during HBV replication.

**HBx upregulates p53 and PA28γ levels to stimulate HBV propagation**

We investigated whether p53-dependent PA28γ upregulation and subsequent HBx elevation eventually affects HBV replication. Previous reports have demonstrated HBx as a positive regulator of HBV replication [6–11, 31]. Consistently, the amount of virus particles released from HepG2 cells transfected with 1.2-mer WT was about 10-fold higher compared with that obtained from cells transfected with 1.2-mer HBx-null (Fig. 5a). HBx also stimulated HBV replication in Hep3B cells, but with much lower efficiency (Fig. 5a), suggesting that HBx also stimulates virus replication in a p53-independent manner(s). Compared with Hep3B cells, HepG2 cells expressed higher levels of viral proteins, such as HBc, HBs and HBx, when transfected with 1.2-mer WT (Fig. 4a), releasing approximately 3.4-fold more virus particles (Fig. 5a). These effects might have resulted from the higher levels of p53 and PA28γ in HepG2 cells (Fig. 4a). Indeed, downregulation of HBx levels as a result of p53 knockdown resulted in a decrease in HBV production in HepG2 cells, whereas upregulation of HBx levels by p53 overexpression led to an increase in HBV production in Hep3B cells (Figs 4d and 5b). Interestingly, this preferential HBV replication in the presence of p53 was not observed in cells transfected with 1.2-mer HBx-null (Fig. 5a), suggesting that HBV proteins other than HBx cannot exploit p53 as a positive regulator of HBV replication.

Finally, we investigated whether upregulation of PA28γ levels is implicated in the stimulation of HBV replication by HBx. Knockdown of PA28γ in HepG2 cells downregulated the levels of intracellular HBV proteins (HBx and HBs) derived from 1.2-mer WT, as well as extracellular HBV particles released from them (Fig. 5c, d). In addition, PA28γ overexpression in Hep3B cells upregulated both intracellular HBx and HBs levels, and extracellular virus particles (Fig. 5c, d). Taken together, we conclude that HBx stimulates HBV replication via p53-dependent activation of PA28γ expression in human hepatoma-derived cell lines.

**DISCUSSION**

Early studies implicated HBx in the development of HCC through its ability to inhibit the potential of p53 to induce apoptosis [33, 34]. For this effect, HBx binds to and sequesters p53 in the cytoplasm to interfere with p53 activities, including sequence-specific DNA binding and transcriptional transactivation [33–36]. HBx is also known to transcriptionally repress the p53 gene in HepG2 cells [37]. However, later studies proposed a completely opposite role for HBx in the regulation of p53, wherein HBx upregulates p53 levels to stimulate the expression of its target genes, such as p21, Siah-1 and pro-apoptotic genes, including Bax, Fas and Nox, which ultimately leads to growth inhibition and apoptotic death of the hepatocytes [25, 28, 29, 38, 39]. The present study consistently showed that HBx upregulates p53 levels to upregulate PA28γ levels in human hepatoma-derived cell lines. It is not easy to reconcile the apparently opposite effects of HBx on p53. Several factors, including cell context, HBx variation, expression levels and other experimental conditions, may affect the interaction between HBx and p53 [29, 40]. In addition, little is known of how and why HBx upregulates p53 and PA28γ levels during HBV replication.

PA28γ is implicated in the Ub-independent degradation of unneeded or damaged proteins as an essential component of the 20S proteasome [14–16]. Clinical studies have demonstrated frequent PA28γ overexpression in human cancers, including HCC [22]; however, the underlying mechanism and its role in HBV replication are poorly understood. The present study provides several lines of evidence supporting the view that HBx activates PA28γ expression via upregulation of p53 levels in human hepatoma cells. First, HBx increased the promoter activity and protein level of PA28γ in HepG2 cells, whereas these effects were undetectable in p53-negative Hep3B cells (Figs 1a and 2d). Consistently, the PA28γ promoter with mutations
in the p53REs was little affected by HBx in HepG2 cells (Fig. 2c). In addition, HBx enhanced p53 binding to the p53REs of the PA28γ promoter in HepG2 cells (Fig. 2e). Moreover, p53 knockdown almost completely abolished the potential of HBx to activate PA28γ expression in HepG2 cells, whereas ectopic p53 expression completely restored this ability of HBx in Hep3B cells (Figs 1c and 2d). Lastly, introduction of the 1.2-mer HBV replicon, but not its HBx-null counterpart, resulted in activation of PA28γ expression via upregulation of p53 levels in HepG2 cells, but not in Hep3B cells (Figs 4a and 5c). Interestingly, the core protein of hepatitis C virus (HCV) also activates PA28γ expression via upregulation of p53 levels [41]. Considering the ability of PA28γ to modulate cell proliferation and apoptosis, primarily by promoting degradation of cell cycle regulatory proteins, including p14, p16, p21 and p53 [17–19, 42], HBx and the HCV core protein may exploit their oncogenic potential via a common route, i.e. activation of PA28γ expression.

There is growing evidence supporting the notion that proteasomes provide potent anti-viral defence systems, restricting virus replication via degradation of essential viral proteins. For example, E6AP, an E3 Ub ligase of the HCV core protein, acts as a negative regulator of HCV propagation by inducing Ub-dependent proteasomal degradation of the HCV core protein [43]. Another E3 Ub ligase, Siah-1, similarly inhibits HBV replication by inducing Ub-dependent proteasomal degradation of HBx [24, 44]. Some viruses, including HBV, have evolved strategies to block or redirect the proteasome to maximize their chance to produce infectious virus particles [27, 45–47]. The first and best example of this came from a study of human papilloma virus type 16 (HPV-16) and HPV-18, which utilize their nonstructural protein, E6, in conjunction with E6AP, to induce the Ub-dependent proteasomal degradation of p53 [48]. In addition, the HCV core protein inhibits E6AP expression to escape from Ub-dependent proteasomal degradation, resulting in stimulation of virus propagation [49].
The present study clearly demonstrated that HBx activates PA28γ expression to upregulate its own protein level and thereby stimulate HBV propagation. Both the HBx levels and HBV replication rates were higher in HepG2 cells in which HBx activated PA28γ expression than in Hep3B cells, in which PA28γ levels were little affected by HBx (Figs 1a, 4a and 5a). In addition, PA28γ knockdown dramatically downregulated HBx levels in HepG2 cells, resulting in a decrease in HBV replication, whereas ectopic PA28γ expression in Hep3B cells upregulated HBx levels, resulting in an increase in HBV replication (Figs 3d, f and 5d).

Fig. 5. HBx stimulates HBV replication by activating PA28γ expression. (a) HepG2 and Hep3B cells were transiently transfected with either 1.2-mer WT or its HBx-null derivative for 48 h. Levels of HBV particles released from the cells were determined by IP-coupled conventional PCR and real-time PCR (n=5). (b) Levels of HBV particles released from the cells prepared in Fig. 4(d) were determined as above (n=3). (c) HepG2 and Hep3B cells were transfected with either an empty vector or 1.2-mer WT, along with the indicated amount of the PA28γ shRNA plasmid or PA28γ expression plasmid, followed by Western blotting. (d) Levels of HBV particles released from the cells prepared in (c) were determined as above (n=5).

How can PA28γ stabilize HBx to upregulate its protein levels? Several possibilities can be considered. PA28γ could induce p53 degradation by facilitating interactions between MDM2 and p53 [17, 18]. Indeed, PA28γ knockdown in the presence of HBx upregulated both p53 and Siah-1 levels, resulting in an increase in HBx ubiquitination and subsequent downregulation of its intact protein level (Fig. 3d, g). A previous report also demonstrated that PA28γ downregulates E6AP levels to protect the HCV core protein from Ub-dependent proteasomal degradation, thereby stimulating virus propagation [50]. Therefore, it is possible that PA28γ downregulates p53 levels via a negative feedback loop to protect HBx from the Siah-1-mediated ubiquitination and subsequent proteasomal degradation. The present study also showed that overexpression of PA28γ in the absence of p53 upregulated HBx levels without affecting Siah-1 levels and HBx ubiquitination (Fig. 3f, g), suggesting that PA28γ also inhibits Ub-independent proteasomal degradation of HBx. However, this is most unlikely when considering the established role of PA28γ as an activator of proteasomes in the Ub-independent degradation of substrates [14–16]. Instead, the increased PA28γ may simply disturb the assembly of the 26S proteasome required for the Ub-dependent proteasomal degradation of HBx by consuming the 20S catalytic core in the construction of the 20S proteasome. More detailed studies are required to reveal the mechanism by which PA28γ stabilizes HBx in human hepatocytes.

On the basis of the present study and others, we propose a regulatory circuit involving HBx, p53 and PA28γ (Fig. 6). According to this model, the circuit starts to work as HBx upregulates p53 levels. HBx directly interacts with voltage-dependent anion channel 3 in mitochondria to increase the intracellular reactive oxygen species level [51, 52]. HBx is also known to induce the accumulation of γ-H2AX, a...
HBx activates p53 via modulation of DNA damage signalling pathways. Consistently, HBx successively activates ATM and CHK2 via their phosphorylation at the Ser-1981 and Thr-68 residues, respectively, which leads to activation of p53 via its phosphorylation at the Ser-15 and Ser-20 residues [24, 53]. The increased p53 protein activates Siah-1 expression to induce Ub-dependent proteasomal degradation of HBx, as demonstrated by our recent report [24]. According to this report, Siah-1 knockdown in HepG2 decreases the ubiquitinated forms of HBx, resulting in upregulation of HBx levels, whilst Siah-1 overexpression in Hep3B cells increases the ubiquitinated forms of HBx, resulting in downregulation of HBx levels, confirming the role of Siah-1 as a negative regulator of HBx. Increased p53 also activates PA28γ expression to upregulate HBx levels, resulting in stimulation of HBV replication, as demonstrated in the present study. Therefore, HBx regulates its own level by triggering positive and negative feedback loops mediated by PA28γ and Siah-1, respectively. According to the results of the present study, HBx levels were higher in the presence of p53 (Fig. 3b). In addition, p53 knockdown downregulated PA28γ and HBx levels although it downregulated Siah-1 levels [24] and thereby decreased the ubiquitinated forms of HBx in HepG2 cells (Fig. 3g) in HepG2 cells, whereas ectopic p53 expression in Hep3B cells upregulated both PA28γ and HBx levels although it upregulated Siah-1 levels [24] and thereby increased the ubiquitinated forms of HBx in HepG2 cells (Fig. 1c). Therefore, the PA28γ-mediated feedback loop appears to be dominant over the Siah-1-mediated one in the regulation of HBx. However, several factors, including p53 status, HBx variations, the status of host proteasomes and their activities, especially those mediated by PA28γ and Siah-1, and other experimental conditions, might affect the HBx level during HBV replication.

The regulatory circuit may also contribute to the creation of an environment favourable for HBV replication by maintaining optimum levels of p53, PA28γ and HBx. The present study showed that HBV replicated more effectively in HepG2 cells in which HBx upregulated its own level via activation of p53 (Fig. 5a). In addition, p53 knockdown slowed HBV replication in HepG2 cells (Fig. 5b), whereas ectopic p53 expression increased it in Hep3B cells (Figs 1c and 5b), indicating that p53 serves a positive regulator of HBV replication. PA28γ in the regulatory circuit might prevent the accumulation of p53 to a level that can cause abortive virus replication. In addition, PA28γ could indirectly interfere with the functions of p53 as a negative regulator of cell growth by promoting degradation of cell cycle regulators, including p14, p16 and p21 [19, 20]. The regulatory circuit might also be required to strictly control virus particle production and thereby allow HBV to evade host defences, such as an immune response and programmed cell death, which can lead to abortive infection.

**METHODS**

**Plasmids**

The HBx expression plasmid, pCMV-3 × HA1-HBX3, encoding HBx downstream of three copies of the influenza virus haemagglutinin (HA) epitope, was described previously [29]. The HBV replicon, 1.2-mer WT, containing 1.2 units of the HBV genome (genotype D) and its HBx-null counterpart (1.2-mer HBx-null) were described previously [31]. The reporter plasmid PA28γ-luc, containing the PA28γ promoter region from −1039 to +109, and its derivative PA28γmp53RE-luc, in which the three p53 REs were point-mutated, were described previously [41]. The PA28γ expression plasmid pCMV6 PSME3, encoding full-length human Myc-DDK-tagged PA28γ, and the human NTCP expression plasmid were purchased from OriGene. SC shRNA, p53 shRNA and PA28γ shRNA plasmids were purchased from Sigma. Plasmid pCH110, encoding the *Escherichia coli* β-Gal gene under the control of the SV40 promoter, was obtained from Pharmacia. Plasmids pHA-Ub, encoding HA-tagged Ub and pCMV p53-WT were gifts from Y. Xiong (University of North Carolina at Chapel Hill, USA) and C.W. Lee (Sungkyunkwan University, Korea), respectively.

**Cell culture and luciferase assay**

HepG2 (KCLB No. 58065) and Hep3B (KCLB No. 88064) cells were obtained from the Korean Cell Line Bank. For transient expression, 2 × 10⁵ cells per 60 mm dish were transfected with the indicated amount of plasmid(s) and an empty vector supplemented to make the final amount of cocktails equal, using WelFect-EX PLUS (WelGENE), according to the manufacturer’s instructions. Stable cell lines, HepG2-vector, HepG2-HBx and Hep3B-HBx, were
established by transfection with pCMV-3×HA1 and pCMV-3×HA1-HBX3, respectively, followed by selection with 500 µg ml⁻¹ of G418 (Gibco). If necessary, cells were treated with 100 µM MG132 (Sigma) for 4 h before harvesting. For the luciferase assay, 500 ng of a reporter plasmid was cotransfected with either an empty vector or an effector plasmid under the indicated conditions. Overall, 100 ng of pCH110 were also included as an internal control. Then, 48 h later, the luciferase assay was performed using a Luciferase Reporter 1000 Assay System (Promega), and values obtained were normalized to that of the β-Gal activity measured in the corresponding cell extracts.

**HBV replicon system**

Cells were transiently transfected with either 1.2-mer WT or its HBx-null mutant for 48 h, as described above. For the preparation of virus stocks, the HBV-producing cell lines were established by transfection with a 1.2-mer HBV replicon, followed by selection with 500 µg ml⁻¹ G418 (Gibco). Virus titres in cultured medium were determined by real-time PCR, as described below. For HBV infection, HepG2 cells were first transfected with 1 µg of the NTCP expression plasmid for 24 h and then infected with HBV in the serum-free Dulbecco’s modified Eagle’s medium (DMEM, WelGENE) at 10 genome equivalents (GEq) per cell for 2 h. Cells were then washed twice with serum-free DMEM, and maintained for an additional 46 h in DMEM supplemented with 2 % fetal calf serum (FCS, Gibco). Viral infection was analysed by measuring viral DNA and viral antigen expression. Immunoprecipitation (IP)-coupled PCR was performed to measure the levels of extracellular HBV particles. Briefly, HBV particles were immunoprecipitated from the culture supernatant using an anti-HBs antibody (Santa Cruz Biotechnology), according to the protocol described in the IP section. HBV genomic DNA was purified from the precipitated HBV particle-antibody complexes using the QIAamp DNA mini kit (Qiagen). For conventional PCR analysis of HBV DNA, the genomic DNA was amplified using Solg 2×Taq PCR Smartmix1 (Solgent) and a primer pair, HBV 1399F (5’-TGG TAC CTC CGC GGG ACG TCC TT-3’) and HBV 1632R (5’-AGC TAG CGT TCA CGG TGT CTC C3’). Quantitative real-time PCR assay (qPCR) of HBV was carried out, as described previously [54]. Briefly, HBV DNA was amplified using the SYBR premix Ex Taq II (Takara Bio) and a primer pair, HBV 379F (5’-GTG TCT GCG GCG TTT TAT CA-3’) and HBV 476R (5’-GAC AAA CCG GCA ACA TAC CTT-3’) in a Rotor Gene Q PCR machine (Qiagen).

**Western blotting**

Cells were lysed in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 % SDS and 1 % NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with antibodies against p53, HBS, PA28γ, Siah-1 and Ub (Santa Cruz Biotechnology); HBx (Millipore); and γ-tubulin (Sigma), and subsequently with an appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse, anti-goat or anti-rabbit IgG (H+L)-HRP (Bio-Rad). An ECL kit (Amersham) was used to visualize the protein bands via the ChemiDoc XRS imaging system (Bio-Rad).

**IFA**

Cells grown on coverslips were fixed in 4 % formaldehyde at room temperature for 15 min and then permeabilized in methanol at −20 °C for 10 min. The HBx and PA28γ proteins were reacted with anti-HBx (Millipore) and anti-PA28γ (Santa Cruz Biotechnology) antibodies overnight at 4 °C and then with anti-mouse IgG-fluorescein isothiocyanate (FITC) and anti-rabbit IgG-rhodamine antibodies (Santa Cruz Biotechnology), respectively, at room temperature for 1 h. Slides were prepared with UltraCruz mounting medium (Santa Cruz Biotechnology) and then visualized under an Eclipse fluorescence microscope (Nikon).

**IP**

An IP assay was performed using a Classic Magnetic IP/Co-IP assay kit (Pierce), according to the manufacturer’s specifications. Briefly, 2 × 10⁶ cells were transfected with pHA-Ub for 48 h under the indicated conditions. The whole cell lysates were incubated overnight at 4 °C with an anti-HA antibody (Santa Cruz Biotechnology). Protein A/G magnetic beads (Pierce) were then added, and the lysates were incubated for an additional 1 h. The beads were collected using a magnetic stand (Pierce), and the antigen/antibody complexes eluted were subjected to Western blotting using an anti-HBx antibody (Millipore).

**ChIP assay**

The ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology), according to the manufacturer’s instructions. The sheared chromatin was immunoprecipitated with either an anti-p53 antibody or human IgG (Santa Cruz Biotechnology). DNA released from the precipitated complexes was amplified to detect the PA28γ promoter by PCR using a primer pair (forward, 5’-CGC ACT GGA TTT TGA AGA CCTT-3’; reverse, 5’-CGA GGC TCA AGT GTTT TAG GC-3’), as previously described [26].

**PCR**

For conventional RT-PCR, total RNA (3 µg) extracted from cells using an RNeasy Mini Kit (Qiagen), was reverse transcribed with the corresponding reverse primer. One-quarter of the reverse transcribed RNA was amplified with Taq polymerase to measure PA28γ, HBx and GAPDH RNA levels, as previously described [41, 55]. Quantitative real-time RT-PCR of PA28γ RNA was performed using a Rotor-Gene Q (Qiagen) with SYBR Green PCR master mix (Takara), as previously described [56].

**Statistical analysis**

Values are indicated as means ± SD from at least three independent experiments. A two-tailed Student’s t-test was
performed for all statistical analyses. A P-value of <0.05 was considered to indicate statistical significance.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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