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Bile acids increase hepatitis B virus gene expression and inhibit interferon-α activity

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Hepatitis B virus (HBV) is a 3.2 kb DNA virus that preferentially replicates in the liver. A number of transcription factors, including nuclear receptors, regulate the activities of HBV promoters and enhancers. However, the association between these metabolic events and HBV replication remains to be clearly elucidated. In the present study, we assessed the effects of bile acid metabolism on HBV gene expression. Conditions associated with elevated bile acid levels within the liver include choleostatic liver diseases and an increased dietary cholesterol uptake. The results obtained in the present study demonstrate that bile acids promote the transcription and expression of the gene for HBV in hepatic cell lines; in addition, farnesoid X receptor α and the c-Jun N-terminal kinase/c-Jun signal transduction pathway mediate the regulatory effect of bile acids. Furthermore, an orphan nuclear receptor, small heterodimer partner protein, is also involved in the bile acid-mediated regulation of HBV gene expression. The bile acid-mediated promotion of HBV gene expression counteracts the antiviral effect of interferon-α.

Introduction

Hepatitis B virus (HBV) infection is a major worldwide health problem, with more than 350 million chronically infected individuals who are currently at risk of developing severe liver diseases, including acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma [1–3]. HBV is a 3.2 kb DNA virus, which replicates almost exclusively in the liver and harbors four overlapping ORFs encoding for the surface antigens (preS1, preS2 and S proteins), core antigens (preC and C proteins), reverse transcriptase (P protein) and transactivator (X protein). These genes are under the control of the preS, S, preC, pregenomic and X promoters. Transcription from these promoters is regulated via two enhancer regions, designated as EnhI and EnhII [3–6]. In previous studies, a variety of transcription factors, including nuclear receptors (NRs), have been defined as regulators of HBV promoters and enhancers [3,7,8]. A region within EnhI binds multiple transcription activators of the basic leucine zipper family, including CCAAT/enhancer binding proteins (C/EBPs), the activator protein (AP)-1 complex and activating transcription factors (ATFs). Liver-enriched NRs perform a pivotal role in the regulation of the HBV transcriptional program by binding to both EnhI and EnhII [9–11]. Notably, NRs are also key players in metabolic processes occurring in the liver, operating as central transcription factors for key enzymes associated with gluconeogenesis, lipid metabolism, ketogenesis and cholesterol homeostasis. However, the association between these metabolic events and HBV replication remains to be clearly elucidated. The farnesoid X receptor (FXR) is a metabolic NR expressed in

Abbreviations
AP, activator protein; ATF, activating transcription factor; C/EBP, CCAAT/enhancer binding protein; CDCA, chenodeoxycholic acid; FXR, farnesoid X receptor; HBV, hepatitis B virus; HNF, hepatocyte nuclear factor; IFN-α, interferon α; JNK, c-Jun N-terminal kinase; NR, nuclear receptor; PPAR, peroxisome proliferator-activated receptor; siRNA, small interference RNA.

the liver, intestine, kidney and adipose tissue via the regulation of the expression and function of genes involved in bile acid synthesis, uptake and excretion [12,13]. FXRα- retinoid X receptor α, which has emerged as a key gene involved in the maintenance of cholesterol and bile acid homeostasis, induced an increase in HBV transcription. Under cholestatic conditions, hepatocytes are exposed to increased concentrations of bile acids, resulting in cytopathic effects [14].

In recent studies, bile acids have been shown to inhibit the induction of proteins involved in the antiviral activity of interferon (IFN). This may explain, in part, the lack of responsiveness to IFN therapy in some patients suffering from advanced chronic viral liver diseases [15,16]. In the present study, we hypothesized that bile acids may antagonize antiviral effects of IFNs on HBV through the promotion of HBV transcription and gene expression via the bile acid-mediated pathway. We employed the 1.3× Cp luciferase HBV construct (kindly provided by Y. Shaul, Weizmann Institute of Science, Rehovot, Israel) and the 1.2 mer HBV (HBx^+) replicon and HBV 3xflag (1.2 mer HBV construct including N-terminal 3xflagged HBx kindly provided by W. S. Ryu, Department of Biochemistry, Yonsei University, Seoul, Korea) with the aim of evaluating the effects of bile acids on viral replication. We report that, in the presence of bile acid, the HBx and HBV core protein expression of HBV was significantly increased in the HBV full genome-transfected human hepatoma cell lines. Using an antagonist of bile acid receptor FXR, *z*-guggulsterone, we determined that FXR performs a function in the bile acid-mediated promotion of HBV gene expression. In addition, bile acid-mediated activation of AP-1 (c-Jun/c-Fos) and C/EBPs contributes to the promotion of HBV gene expression. Furthermore, we determined that bile acids compromised the anti-HBV effect of IFN-α in cells. These data suggest a novel mechanism for bile acid-mediated gene regulation in the context of HBV gene expression. Our findings also point to a mechanism that is responsible for the failure of IFN-based treatment in certain HBV patients. Importantly, these studies may contribute to the development of superior regimens for the treatment of chronic HBV infections by including agents that alter the bile acid-mediated FXR and c-Jun N-terminal kinase (JNK)/c-Jun pathways.

Results

Bile acids promote HBV gene expression in human hepatocyte cell lines

Under cholestatic conditions, hepatocytes are exposed to increased bile acid concentrations, resulting in cytopathic effects. These compounds exert direct effects on the cellular, subcellular and molecular levels in both hepatocytes and nonliver cells [17,18]. Additionally, bile acids inhibit the induction of proteins involved in the antiviral activity of IFN [15]. In the present study, we aimed to determine whether HBV transcription and replication might be subject to regulation by bile acids in human hepatoma cells. Cholic acid and chenodeoxycholic acid (CDCA) are two major primary bile acids detected in human bile [19–21]. The effects of bile acids on HBV gene expression were assessed via treatment with different concentrations of unconjugated bile acid, CDCA, in the medium and incubation for different lengths of time (up to 48 h) in human hepatocyte cell lines (Fig. 1). In the Chang liver, HepG2 and Huh7 cells, we observed an increase of the level of 1.3x HBV luciferase activity in a dose-dependent manner after CDCA treatment (Fig. 1A). Similar to that noted for HBV luciferase activity, the mRNA and protein levels of the HBx and HBV core increased in the presence of CDCA incubation in the 1.2 mer HBV replicon-transfected HepG2 cells (Fig. 1B, C, F). In addition, the synthesis of HBV DNA increased in a dose- and time-dependent manner with respect to CDCA treatment (Fig. 1D, E). Collectively, these results show that bile acids increase HBV transcription and gene expression in the 1.2 mer HBV replicon- (including the HBV full genome) transfected human hepatocyte cell lines.

FXR promotes HBV gene expression in human hepatocyte cell lines

The FXR is a metabolic nuclear receptor that is expressed in the liver, intestine, kidney and adipose tissue [22,23]. By regulating the expression and function of genes involved in bile acid synthesis, uptake and excretion, FXR has emerged as a key gene involved in the maintenance of cholesterol and bile acid homeostasis [13,24]. There are two known FXR genes, which are commonly referred to as FXRα and FXRβ; the principal form expressed in the liver is the FXRα [12,13,25]. To determine how bile acids promote HBV transcription and gene expression in human hepatoma cells, the effects of FXRα (FXRα1 and FXRα2) on CDCA-mediated gene expression were assessed in Chang liver and HepG2 cells (Fig. 2). HBV transcriptional activity, mRNA and protein levels were increased by the mFXRα1 expression plasmids (Fig. 2A–C). In addition, mRNA levels of the HBx and HBV core increased in the presence of mFXRα1 and additively after incubation of CDCA in HepG2 cells (Fig. 2F). Furthermore, to determine

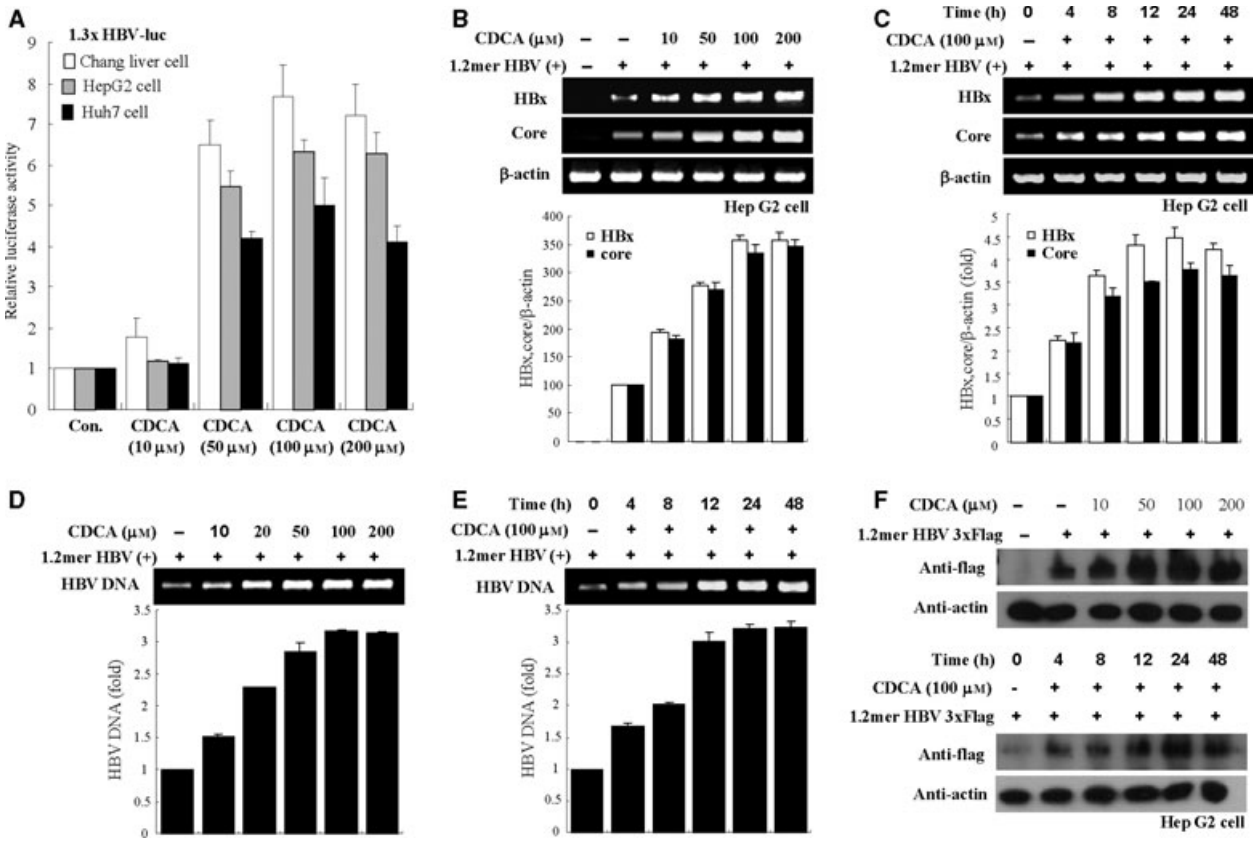

Fig. 1. The effects of bile acids on HBV gene expression in hepatocyte cell lines. (A) Chang liver, HepG2 and Huh7 cells were transfected with the 1.3x HBV-luc construct and maintained either under control conditions or in the presence of different concentrations of unconjugated bile acid, CDCA, for 24 h. (B) HepG2 cells were transfected with 1.2 mer HBV(+) construct and then maintained either under control conditions or in the presence of different concentrations for 24 h. Total RNA was prepared from the cells and the HBx and HBV core mRNA levels was assessed via RT-PCR. The values are expressed as the mean ± SD (n = 4). (C) HepG2 cells were transfected with 1.2 mer HBV(+) construct and then maintained either under control conditions or in the presence of CDCA (100 μM) for different periods of time (up to 48 h). Values are expressed as the mean ± SD (n = 4). The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ, version 1.35d (National Institutes of Health). (D) HepG2 cells were maintained either under control conditions or in the presence of different concentrations of CDCA for 24 h. Total DNA was prepared from the cells and the HBV DNA levels was detected by PCR. The DNA bands were quantified with ImageJ, version 1.35d (National Institutes of Health). (E) HepG2 cells were maintained either under control conditions or in the presence of CDCA (100 μM) for different periods of time (up to 48 h). (F) HepG2 cells were transfected with HBV 3xflag construct and maintained either under control conditions or in the presence of different concentrations of CDCA for 24 h. Forty-eight hours after transfection, western blotting was performed on the cell extracts using anti-Flag serum. The equivalence of protein loading in the lanes was verified by the anti-actin serum.

whether mFXRα1 mediates bile acid-induced HBV gene expression, we tested an antagonist of FXR, *z*-guggulsterone (10 μM), and siFXR (Fig. 2E) in the presence of CDCA (100 μM) or mFXRα1. As predicted, 12 h of treatment with *z*-guggulsterone (10 μM) reduced HBV transcriptional activity (Fig. 2D) and the expression of HBx, HBV core mRNA level (Fig. 2G). These results reveal that FXRα1 plays important roles in both HBV transcription and gene expression.

The JNK/c-Jun pathway mediates HBV gene expression in human hepatocyte cell lines

Previous studies of human HBV transcription revealed the requirement of two enhancer elements, named EnhI and EnhII [4,7,26]. However, the activity of EnhII depends on a functional EnhI. EnhI is located upstream of the X promoter and is targeted by multiple activators, including, C/EBPs, AP-1 complex and ATFs. Recently, it was reported that a physiologic

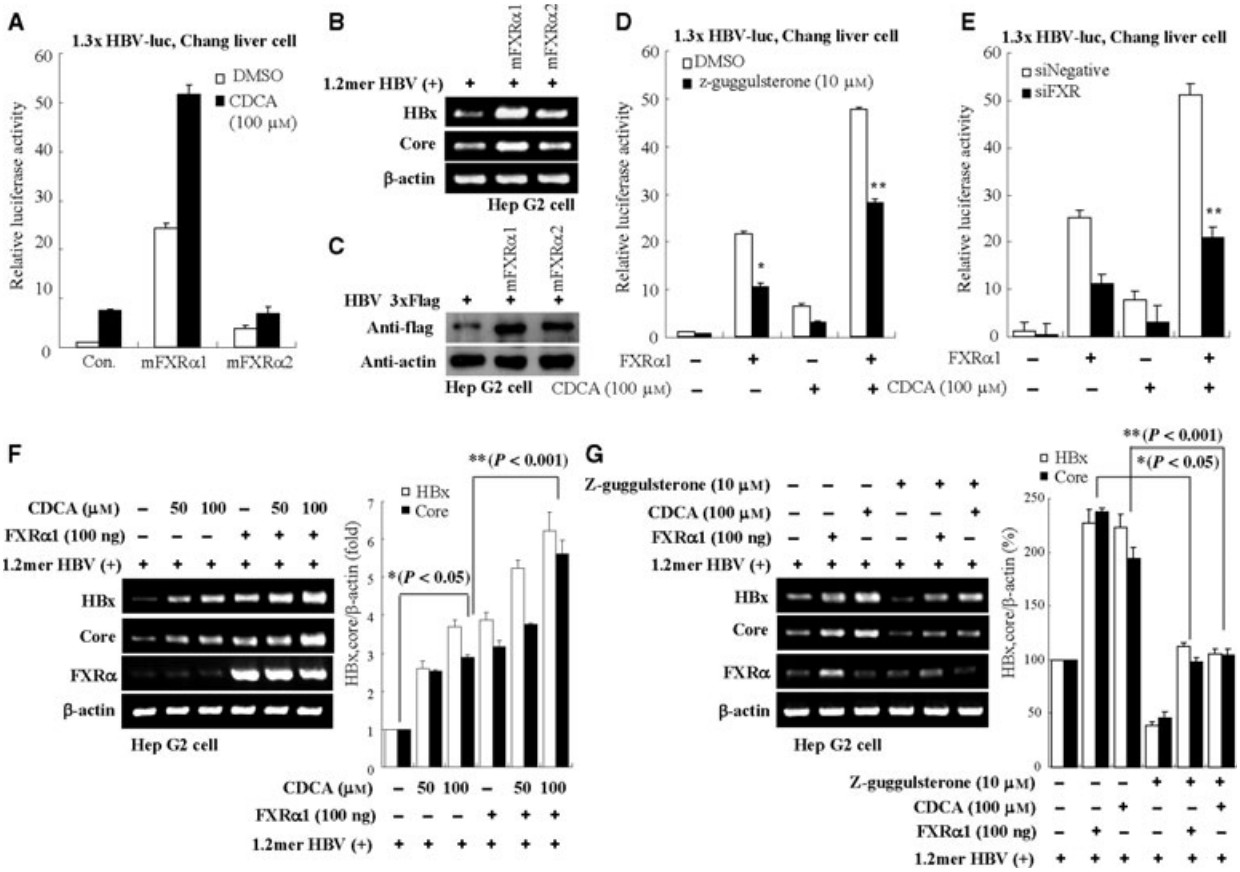

Fig. 2. The effects of FXRα1 on HBV gene expression in hepatocyte cell lines. (A) Chang liver cells were cotransfected with the 1.3x HBV-luc construct and the indicated plasmids, and then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. (B) HepG2 cells were cotransfected with the 1.2 mer HBV(+) construct and the indicated plasmids. Total RNA was prepared from the cells and the HBx and HBV core mRNA levels were assessed via RT-PCR. (C) HepG2 cells were cotransfected with the HBV 3xflag construct and the indicated plasmids. Western blotting was performed on the cell extracts using anti-Flag serum. The equivalence of protein loading in the lanes was verified using anti-actin serum. (D) Chang liver cells were cotransfected with the 1.3x HBV-luc construct and the FXRα1 expression plasmid or treated with CDCA (100 μM) for 24 h. The cells were then maintained either under control conditions or in the presence of *z*-guggulsterone (10 μM) for 12 h (**P* < 0.05 and ***P* < 0.01 compared to mock transfectants). (E) For the siRNA-mediated downregulation of FXR, negative control siRNA or FXR-specific siRNA was transfected with or without CDCA (100 μM) into Chang liver cells. The transfected cells were analyzed by luciferase assay. (F) HepG2 cells were cotransfected with the 1.2 mer HBV(+) construct and the FXRα1 expression plasmid and maintained either under control conditions or in the presence of CDCA (50, 100 μM) for 24 h. (G) HepG2 cells were cotransfected with 1.2 mer HBV(+) construct and the FXRα1 expression plasmid or treatment with CDCA (100 μM) for 24 h. The cells were maintained either under control conditions or in the presence of *z*-guggulsterone (10 μM) for 12 h. Total RNA was prepared from the cells and the HBx and HBV core mRNA levels and then the FXRα mRNA levels were determined via RT-PCR. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band using ImageJ, version 1.35d (National Institutes of Health).

concentration of bile acids could cause activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway [27], JNK pathway and p38 pathway [28–30]. As a result of the findings described above, we determined whether the basic leucine zipper transcription factors AP-1 (c-Jun) and C/EBPs, which are downstream of mitogen-activated protein kinase signaling, participated in bile acid-induced HBV gene expression (Fig. 3A, B). ATF2 and cAMP response element binding protein, which are recently reported to be associated with HBV replication, were used as a positive control [30,31]. As shown in Fig. 3A, CDCA treatment significantly increased the FXRα1-induced transactivation of AP-1 and the C/EBP responsive element of reporters. In addition, ectopic expression of C/EBPα, C/EBPβ, ATF2, c-Jun, c-Fos and cAMP response element binding protein enhanced HBV gene expression, and additional treatment with CDCA increased the transactivation (Fig. 3 B). To further confirm the regulatory roles of c-Jun

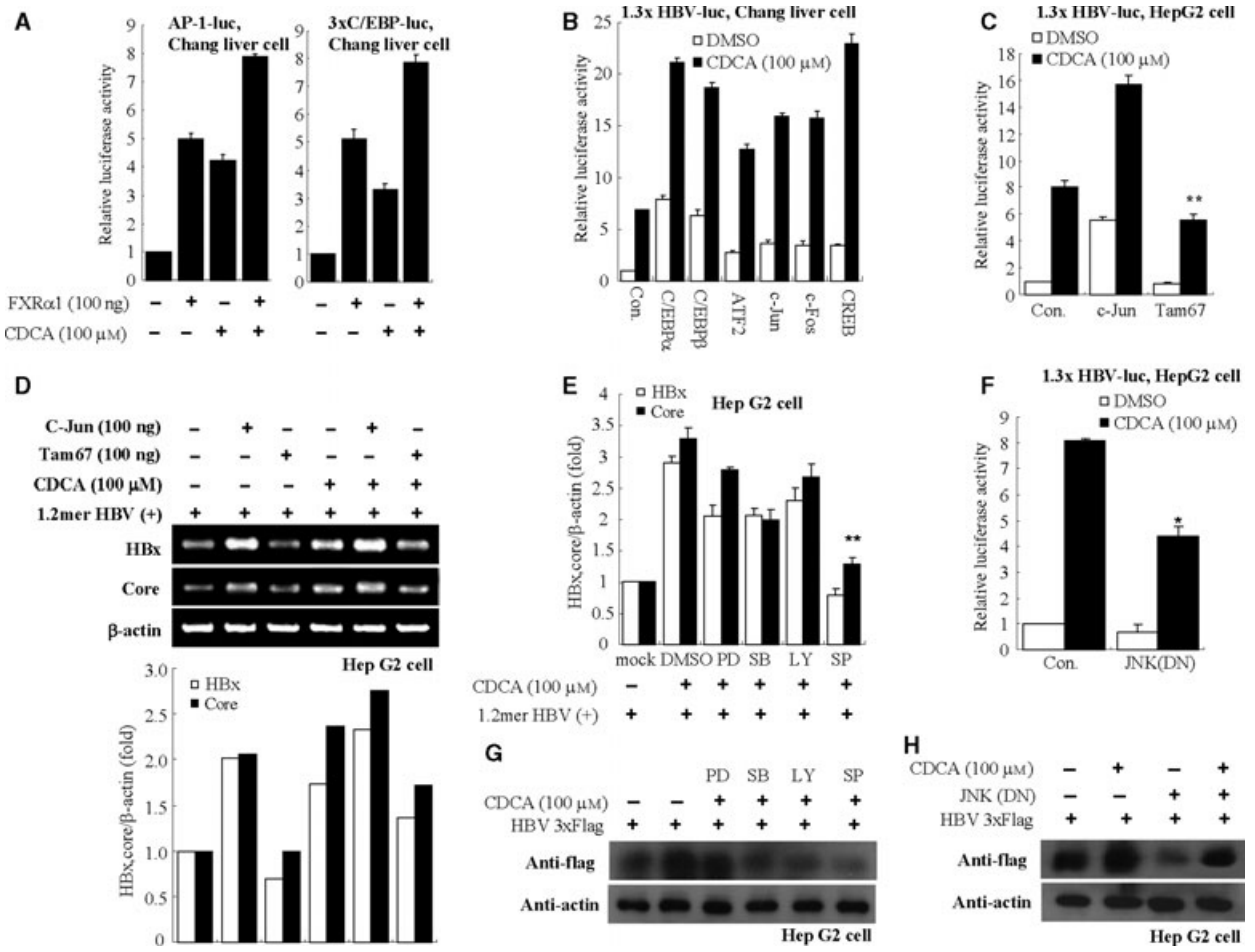

Fig. 3. The effect of AP-1 and C/EBPs on bile acids-induced HBV gene expression. (A) Chang liver cells were cotransfected with AP-1-luc or 3xC/EBP-luc construct and the indicated plasmids, FXRα1. The cells were then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. (B) Chang liver cells were cotransfected with 1.3x HBV-luc construct and the indicated plasmids. The cells were then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. (C) HepG2 cells were cotransfected with 1.3x HBV-luc construct and the indicated c-Jun or Tam67 plasmid. The cells were then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. (D) HepG2 cells were cotransfected with 1.2 mer HBV(+) construct and the indicated plasmids. Then the cells were maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. The transfected cells were analyzed by RT-PCR. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ, version 1.35d (National Institutes of Health Image). (E) HepG2 cells were cotransfected with 1.2 mer HBV(+) construct. Then the cells were maintained either under control conditions or in the presence of CDCA (100 μM) and various pharmacological protein kinase inhibitors for 24 h. The transfected cells were analyzed by RT-PCR. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ, version 1.35d. The values are expressed as the mean ± SD ($n = 3$) (**$P < 0.01$ compared to mock transfectants). (F) HepG2 cells were cotransfected with 1.3x HBV-luc construct and the JNK(DN) plasmids. The cells were then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h (*$P < 0.05$ compared to mock transfectants). (G) HepG2 cells were cotransfected with HBV 3xflag construct. Then the cells were maintained either under control conditions or in the presence of CDCA (100 μM) and various pharmacological protein kinase inhibitors for 24 h. The transfected cells were analyzed by western bloting. (H) HepG2 cells were cotransfected with HBV 3xflag construct and the JNK(DN) plasmids. The cells were then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. The transfected cells were analyzed by western blotting.

in CDCA-induced HBV gene expression, the deleted construct of c-Jun (Tam67), which can act as a dominant negative mutant against the full-length c-Jun, was used for a HBV gene expression assay. As predicted, transfection of Tam67 significantly reduced the transcriptional activity of HBV (Fig. 3C), as well as the expression of HBx and HBV core mRNA (Fig. 3D), compared to c-Jun. Next, to determine which kinase is

necessary for HBV gene expression after CDCA treatment, a series of protein kinase inhibitors were subjected to a gene transcription study. HepG2 cells were treated with 100 μM CDCA and maintained in the presence of pharmacological protein kinase inhibitors, 25 μM PD98058 (extracellular signal-regulated kinase inhibitor), 20 μM SB203580 (p38 kinase inhibitor), 20 μM LY294002 (PI3K inhibitor) and 20 μM SP600125 (JNK inhibitor). The results obtained indicate that JNK inhibitor (i.e. SP600125) significantly reduced the expression of HBx and HBV core mRNA (Fig. 3E) and protein levels (Fig. 3G), suggsting that JNK-mediated phosphorylation of key transcription factors is involved in CDCA-induced HBV expression. This was confirmed using the JNK dominant-negative construct (Fig. 3F,H). These results demonstrate that The CDCA-induced JNK/c-Jun pathway cooperates with the FXR pathway in the promotion of HBV transcription and gene expression.

The small heterodimer partner (SHP) inhibits HBV gene expression in human hepatocyte cell lines

SHP is abundant in the liver, where it performs a crucial function in cholesterol metabolism by modulating the transcription of enzymes involved in the pathway converting cholesterol into bile acids, and it is also induced by FXR [19,32]. SHP is a unique orphan nuclear receptor that lacks a conserved DNA binding domain but harbors a receptor-interacting domain and a repressor domain [19,33]. SHP has been shown to inhibit the transactivation activity of retinoic acid receptor (RXR), hepatocyte nuclear factor (HNF)4α, peroxisome proliferator-activated receptor (PPAR) and thyroid hormone receptor [34], which are well known potent activators of HBV promoters and enhancers. To determine whether bile acid-induced SHP expression affects the induction of HBV gene expression by the bile acid-induced FXRα pathway, Chang liver cells were transfected with the expression vector encoding for HA/SHP in the presence of CDCA (100 μM) or FXRα1 along with the 1.3x HBV luciferase reporter (Fig. 4A). The mRNA levels of the HBx and HBV core were confirmed via RT-PCR (Fig. 4B). In an attempt to obtain additional insight into the role of SHP with respect to the inhibition of HBV gene expression, loss-of-function studies were conducted using a small interference RNA (siRNA) approach. We observed that the knockdown of SHP gave rise to an increase in transcriptional activity, mRNA and protein levels of HBV in the presence of CDCA (100 μM) or FXRα1 (Fig. 4C–E). These results demonstrate that SHP inhibits bile acid/FXRα-induced HBV transcription and gene expression.

Bile acids compromise the anti-HBV effect of IFN-α in human hepatocyte cell lines

IFNs are secreted proteins that are involved in many biological activities, including antiviral defense. In previous studies, bile acids were shown to inhibit the IFN-induced antiviral effect in a concentration-dependent manner [15]. However, the manner in which the anti-HBV effect of IFN is regulated at the molecular level remains unknown. Consequently, we determined whether the anti-HBV effect of IFN-α might be subject to regulation by the bile acid-mediated FXRα or JNK/c-Jun pathways in human hepatoma cells. As shown in Fig. 5, with the aim of characterizing the effect of bile acids on the anti-HBV effect of IFN-α, Chang liver (Fig. 5A, D) and HepG2 cells (Fig. 5B, C, E–G) were treated with IFN-α in the presence or absence of CDCA (100 μM) and indicated gene constructs. After incubation, HBV transcriptional activity, mRNA and protein levels of the HBV viral proteins (HBx and core) were assessed. The relative expression levels of HBV protein or genome affected by IFN-α with or without bile acids were compared with those observed in a mock treatment. As shown in Fig. 5A–C, bile acid compromised the antiviral effect of IFN-α with respect to transcriptional activity, mRNA and protein levels, as expected. Although the bile acid-induced FXRα and JNK/c-Jun pathways interfered with the antiviral effect of IFN-α with respect to transcriptional activity and mRNA levels (Fig. 5D–F), SHP assisted the antiviral effect of IFN-α (Fig. 5G). Collectively, these results indicate that bile acid-induced dysregulation of the FXRα, SHP and JNK/c-Jun pathways may be associated with the failure of IFN-α treatment in HBV-infected cells.

Discussion

In terms of regulation and the response to nutritional stimuli, HBV is quite reminiscent of metabolic genes; thus, one can attribute certain dynamic changes in the natural history of HBV not only to certain mutations or the genotypic diversity of the virus, but also to alterations in environmental nutritional conditions, or alternatively, to preexisting pathologic states that influence the host metabolism [35]. According to previous studies, liver-enriched NRs play a pivotal role in the regulation of the HBV transcriptional program by binding to both EnhI and EnhII via the NR-response

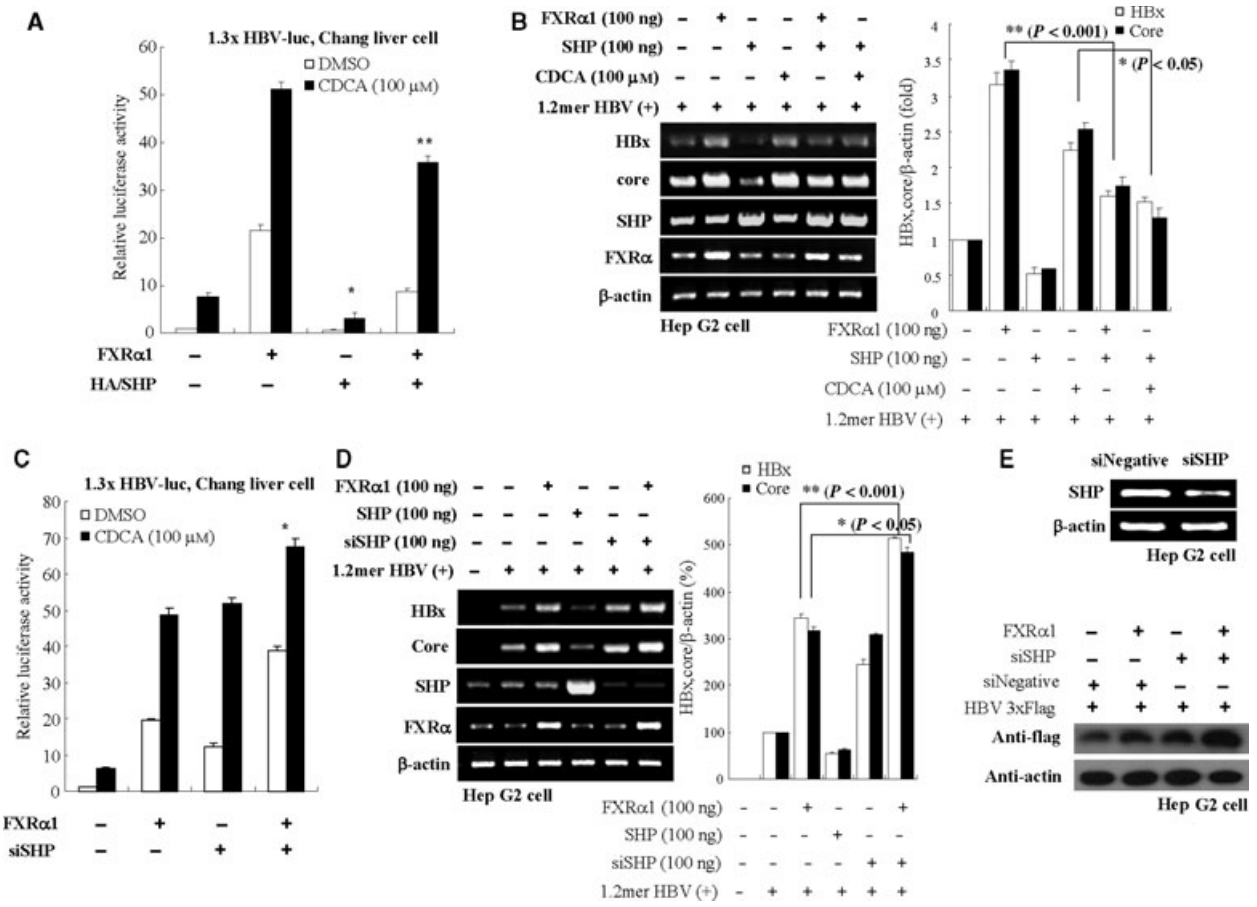

Fig. 4. The effects of SHP on bile acids-induced HBV gene expression in hepatocyte cell lines. (A) Chang liver cells were cotransfected with 1.3x HBV-luc construct and the indicated plasmids. The cells were then maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h (*$P < 0.05$ and **$P < 0.01$ compared to mock transfectants). (B) HepG2 cells were cotransfected with 1.2 mer HBV(+) construct and the indicated plasmids. Then the cells were maintained either under control conditions or in the presence of CDCA (100 μM) for 24 h. Total RNA was prepared from the cells and the HBx, HBV core, SHP and FXRα mRNA levels were detected via RT-PCR. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ, version 1.35d (National Institutes of Health Image). The values are expressed as the mean ± SD ($n = 3$). (C) Chang liver cells were cotransfected with 1.3x HBV-luc construct and the indicated plasmids. For the siRNA-mediated downregulation of SHP, negative control siRNA or SHP-specific siRNA was transfected under control conditions or in the presence of CDCA (100 μM) for 24 h (*$P < 0.05$ compared to mock transfectants). (D) HepG2 cells were cotransfected with 1.2 mer HBV(+) construct and the indicated plasmids. For the siRNA-mediated downregulation of SHP, negative control siRNA or SHP-specific siRNA was transfected under control conditions or in the presence of CDCA (100 μM) for 24 h. Total RNA was prepared from the cells and the HBx, HBV core, SHP and FXRα mRNA levels were assessed via RT-PCR. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ, version 1.35d. The values are expressed as the mean ± SD ($n = 3$). (E) HepG2 cells were cotransfected with HBV 3xflag construct and the indicated plasmids. For the siRNA-mediated downregulation of SHP, negative control siRNA or SHP-specific siRNA was transfected. The transfected cells were analyzed by western blotting.

element [6,26,36]. Interestingly, liver-enriched NRs are central mediators of metabolic processes in the liver. A prominent example of such a process is gluconeogenesis, which is required for the maintenance of a normal blood glucose level during starvation. NRs, including glucocorticoid receptor, HNF4α and PPARs, bind to and activate the promoter of the phosphoenolpyruvate carboxykinase gene, a key gluconeogenic enzyme. In particular, HNF4α, retinoid X receptor α and PPARα mainly bind to the HBV NR-response elements. The essential function of liver-enriched NRs in HBV gene expression led us to investigate a possible association between major metabolic processes occurring in the liver and HBV gene expression. NRs are also involved in fatty acid β-oxidation, ketogenesis and bile acid homeostasis, which comprise other essential metabolic events occurring in the liver [35,37]. Cholesterol homeostasis is maintained by *de novo* synthesis,

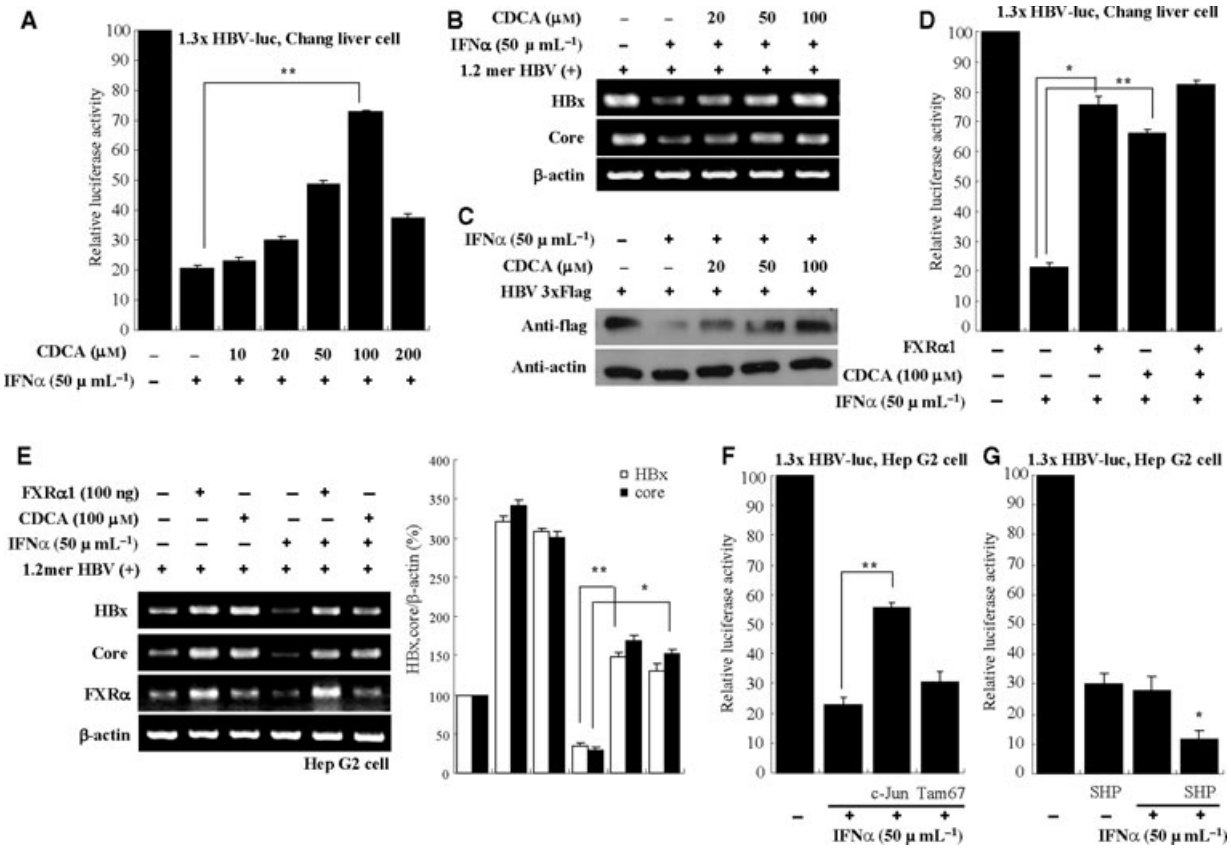

Fig. 5. Bile acids and the anti-HBV effect of IFN-α in hepatocyte cell lines. (A) Chang liver cells were transfected with the 1.3x HBV-luc construct and then incubated with mock-medium, IFN-α alone (50 U·mL^{-1}) or IFN-α with various concentrations of CDCA for 24 h (**P < 0.01 compared to mock transfectants). (B) HepG2 cells were transfected with the 1.2 mer HBV(+) construct and then incubated with mock-medium, IFN-α alone (50 U·mL^{-1}) or IFN-α with various concentrations of CDCA for 24 h. The transfected cells were analyzed by RT-PCR. (C) HepG2 cells were transfected with the HBV 3xflag construct and then incubated with mock-medium, IFN-α alone (50 U·mL^{-1}) or IFN-α with various concentrations of CDCA for 24 h. The transfected cells were analyzed by western blotting. (D) Chang liver cells were cotransfected with 1.3x HBV-luc construct and FXRα1 expression plasmid and then treated with or without CDCA (100 μM) for 24 h. Then the cells were incubated with mock-medium or IFN-α alone (50 U·mL^{-1}) for 12 h (*P < 0.05 and **P < 0.01 compared to mock transfectants). (E) HepG2 cells were cotransfected with the 1.2 mer HBV(+) construct and the FXRα1 expression plasmids and were then treated with or without CDCA (100 μM) for 24 h. Then the cells were incubated with mock-medium or IFN-α alone (50 U·mL^{-1}) for 12 h. The transfected cells were analyzed by RT-PCR. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ, version 1.35d (National Institutes of Health Image). The values are expressed as the mean ± SD (n = 3) (*P < 0.05 and **P < 0.01 compared to mock transfectants). (F) HepG2 cells were cotransfected with 1.3x HBV-luc construct and c-Jun or Tam67 plasmid. Then the cells were incubated with mock-medium or IFN-α alone (50 U·mL^{-1}) for 12 h (**P < 0.01 compared to mock transfectants). (G) HepG2 cells were cotransfected with 1.3x HBV-luc construct and SHP plasmid. Then the cells were incubated with mock-medium or IFN-α alone (50 U·mL^{-1}) for 12 h (*P < 0.05 compared to mock transfectants).

dietary absorption, and catabolism to bile acids and other steroids, as well as excretion into the bile [14]. Cholestasis is a medical condition characterized by an impairment of normal bile flow; this impairment results either from a functional defect of bile secretion, or from an obstruction of the bile duct [38]. Under cholestatic conditions, hepatocytes are exposed to increased bile acid concentrations, resulting in cytopathic effects [14,39]. Recent studies have demonstrated that bile acids not only serve as physiological detergents that facilitate the absorption, transport and distribution of lipid soluble vitamins and dietary fats, but also as signaling molecules that activate NRs and regulate bile acid and cholesterol metabolism [14,19]. Additionally, it has been demonstrated that bile acids inhibit the induction of proteins involved in the antiviral activity of the interferons IFNs [15]. One of the classes of anti-HBV IFNs comprises secreted proteins

that are involved in many biological activities, including antiviral defense [15,40]. Under cholestatic conditions in several environments, and because hepatocytes are exposed to high concentrations of bile acids in the liver [38], we hypothesized that the bile acid-mediated pathway demonstrates regulatory capacities with regard to HBV gene expression and the anti-HBV effects of IFN-α. In the present study, we demonstrate that bile acids, including an unconjugated CDCA, robustly induce HBV transcription and gene expression in human hepatoma cell lines. In addition, we tested whether the bile acid-mediated FXRα pathway is important in bile acid-mediated HBV gene expression using siFXR and the bile acid antagonist FXR, *z*-guggulsterone. This suggests that the FXRα pathway is important for bile acid-mediated HBV gene expression. In recent study, it was reported that two putative FXRE were identified in the EnhII of HBV genome, with homology to the typical inverted repeat sequence recognized by FXRα [41]. These results indicate that the therapeutic inhibition of FXRα with the appropriate antagonist may represent a potential approach for inhibiting HBV gene expression in chronic carriers. Interestingly, the activity of EnhII depends on a functional EnhI. EnhI is located upstream of the X promoter and is targeted by multiple activators, including C/EBPs, AP-1 complex and ATFs. In the present study, we suggest that the CDCA-induced JNK/c-Jun pathway cooperated with the FXRα pathway in the promotion of HBV gene expression. According to previously obtained results [2,4,6,9], we can assume that bile acid-induced HBV gene expression is mediated by the FXRα pathway on EhnII in cooperation with the JNK/c-Jun pathway on Ehn1 of the HBV genome. On the other hand, it has been demonstrated that SHP, an orphan nuclear hormone receptor lacking a DNA binding domain, inhibits NR-mediated transcription and gene expression. The inhibition of HBV replication by SHP is dependent on the presence of NRs [42]. SHP is present abundantly in the liver and performs a crucial function in cholesterol metabolism by modulating the transcription of enzymes involved in the pathway by which cholesterol is converted into bile acids [14]. In the present study, we demonstrate that bile acids, including unconjugated CDCA, which activates the bile acid-mediated FXRα pathway, robustly induce HBV gene expression, whereas increased SHP levels reduce FXRα-induced HBV gene expression in human hepatoma cell lines. The conditions associated with elevated bile acid levels within the liver include choleostatic liver diseases or increased dietary cholesterol uptake [19]. Under these conditions, it was shown that the FXRα and JNK/c-Jun pathways may be elevated. and not only might HBV gene expression consequently be increased, but also the anti-HBV effects of IFNs might be reduced. These observations indicate that the physiological regulation of HBV biosynthesis by bile acids in the liver will depend on both FXRα/JNK-c-Jun pathway levels and the relative inhibition of SHP in the context of HBV gene expression and gene expression. Furthermore, our findings may facilitate the development of novel and superior regimens for the treatment of chronic HBV infections, ostensibly by including agents that alter the bile acid-mediated FXRα and JNK/c-Jun pathways.

Materials and methods

Cell culture

Chang liver, HepG2 and Huh7 cells (all obtained from the American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM with 10% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 1% (v/v) penicillin-streptomycin (Gibco BRL) at 37 °C in a humid atmosphere of 5% CO_2.

Plasmid constructs and reagents

1.3x Cp-luciferase HBV was generously provided by Y. Shaul (Weizmann Institute of Science, Rehovot, Israel) [26,35]. The 1.2 mer HBV (HBx^+) replicon and HBV 3xflag (1.2 mer HBV constructs including N-terminal 3xflagged HBx) were kindly provided by W. S. Ryu [43]. CDCA (sodium salt, 99%) was purchased from Sigma (St Louis, MO, USA) and prepared in dimethylsulfoxide as a 100 mM stock solution. An antagonist of FXR (a nuclear receptor of bile acids), *z*-guggulsterone, was purchased from Sigma and prepared in dimethylsulfoxide as a 50 mM stock solution, respectively. Recombinant human IFN-α2 (Hu-IFNα2) was obtained from PBL Biomedical Laboratories (Piscataway, NJ, USA). The transfection reagents PolyFect and SuperFect were purchased from Qiagen (Hilden, Germany). In studies concerning the effects of protein kinase inhibitors, cells were pretreated with SB203580 (20 μM), PD98059 (25 μM), LY294002 (20 μM) and SP600125 (20 μM) (Calbiochem, San Diego, CA, USA) for 1 h, followed by treatment with CDCA in the presence of the inhibitors.

IFN-α treatment on liver cell lines with or without bile acids

To assess the effects of bile acids on the anti-HBV effects of IFN-α2 (PBL Biomedical Laboratories), Chang liver cells and HepG2 cells were treated with IFN-α in the presence or absence of CDCA or FXRα. One- or 2-day-old semi-confluent cells were incubated with 50 $U \cdot mL^{-1}$ of

IFN-α2 alone or IFN-α2 and various concentrations of CDCA for 24 h. In these studies, we utilized 10, 20, 50, 100 and 200 μM of CDCA. The negative controls included mock-medium or solvent (dimethylsulfoxide).

Transient transfection and luciferase reporter assay

Cells were plated in 24-well culture plates and transfected with luciferase reporter vector (0.2 μg) and β-galactosidase expression plasmid (0.2 μg), together with each indicated expression plasmid using PolyFect (Qiagen). The pcDNA3.1/HisC empty vector was added to the transfections to achieve the same total quantity of plasmid DNA per transfection. After 48 h of transfection, the cells were lysed in the cell culture lysis buffer (Promega, Madison, WI, USA) followed by measurement of luciferase activity. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were conducted at least in triplicate.

siRNA preparation and transient transfection

For the siRNA-mediated downregulation of FXR, SHP-specific siRNA and negative control siRNA were purchased from Bioneer (Daejeon, Korea). The transfection of Chang liver cells and HepG2 cells was conducted using HiPerFect (Qiagen) and jetPEI™ (Polyplus Transfection, Inc., New York, NY, USA) in accordance with the manufacturer's instructions.

RNA isolation and RT-PCR analysis

Total RNA from the transfected Chang liver cells (HepG2 cells) was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Total RNA was converted into single-strand cDNA by Moloney murine leukemia virus reverse transcriptase (Promega) with random hexamer primers. The one-tenth aliquot of cDNA was subjected to PCR amplification using gene-specific primers. HBx: forward primer: 5′-ATG GCTGCTAGGCTGTGCTGC-3′, reverse primer: 5′-ACG GTGGTCTCCATGCGACG-3′; HBV core: forward primer: 5′-ATGCAACTTTTTCACCTCTGC-3′, reverse primer: 5′-CTGAAGGAAAGAAGTCAGAAG-3′; FXRα: forward primer: 5′-GCCTGTAACAAAGAAGCCCC-3′, reverse primer: 5′-CAGTTAACAAGCATTCAGCCAAC-3′; SHP: forward primer: 5′-AGCTATGTGCACCTCATC GCACCTGC-3′, reverse primer: 5′-CAAGCAGGCTGGT CGGAATGGACTTG-3′; and β-actin: forward primer: 5′-GACTACCTCATGAAGATC-3′, reverse primer: 5′-GAT CCACATCTGCTGGAA-3′. The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with IMAGEJ, version 1.35d (National Institutes of Health, Bethesda, MD, USA).

Detection of HBV DNA by PCR

1.2 mer HBV(+) transfected liver cell lines with or without bile acids were digested with proteinase K, and HBV DNA was isolated using Exgene™ Cell SV (GeneAll, Seoul, Korea) in accordance with the manufacturer's instructions. Primer sequences were designed using PRIMER 3 software (J. M. Gao, Central South University, Changsha, China) [23]: forward primer: 5′-TCGGAAATACACCTCCTTTCC ATGG-3′ (HBV genome 1353–1377), reverse primer: 5′-GC CTCAAGGTCGGTCGTTGACA-3′ (HBV genome 1702–1681). The length of the PCR product was 350 bp. Thirty cycles of DNA amplification were conducted in a 50 μL PCR reaction mixture. Each cycle comprised denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, followed by a final 10 min of elongation at 72 °C. The PCR bands were then quantified using IMAGEJ, version 1.35d (National Institutes of Health).

Western blotting and antibodies

Cells were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl (pH 7.5), 1 mM EDTA, 1% Nonidet P-40, 10% glycerol and protease inhibitors for 20 min on ice. The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Fifty micrograms of protein from the whole cell lysates were subjected to 10% SDS-PAGE and transferred to a poly(vinylidene difluoride) membrane (Millipore, Billerica, MA, USA) via semidry electroblotting. The membranes were then incubated for 2 h at room temperature with anti-actin serum (Sigma) or anti-Flag serum (Sigma) in NaCl/Tris Tween supplemented with 1% nonfat dry milk. The bands were detected using an enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

Statistical analyses were conducted using unpaired or paired *t*-tests as appropriate. All data are expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

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