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SREBP-1a activation by HBx and the effect on hepatitis B virus enhancer II/core promoter

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ABSTRACT

Hepatitis B virus (HBV) X protein (HBx) plays an important role in HBV pathogenesis by regulating gene expression. Sterol regulatory element binding protein-1a (SREBP-1a) is a key transcriptional factor for modulating fatty acid and cholesterol synthesis. Here we demonstrated that HBx increased mature SREBP-1a protein level in the nucleus and its activity as a transcription factor. We further showed that the up-regulation of SREBP-1a by HBx occurred at the transcriptional level after ectopic expression and in the context of HBV replication. Deletional analysis using SREBP-1a promoter revealed that the sequence from −436 to −398 in the promoter was required for its activation by HBx. This promoter region possesses the binding sequences for two basic leucine zipper (b-ZIP) transcription factors, namely C/EBP and E4BP4. Mutagenesis of the binding sequences on the SREBP-1a promoter and ectopic expression experiments demonstrated that C/EBPα enhanced SREBP-1a activation by HBx, while E4BP4 had an inhibitory effect. C/EBPα was able to significantly reverse the inhibitory activity of E4BP4 on SREBP-1a promoter. These results demonstrated that HBx activates SREBP-1a activity at the transcription level through a complex mechanism involving two bZIP transcription factors C/EBP and E4BP4 with C/EBP being the dominant positive factor. Finally, we showed that knocking down SREBP-1 abolishes HBV enhancer II/core promoter activation by HBx.

1. Introduction

Sterol regulatory element-binding proteins (SREBPs) belong to the family of basic-helix–loop–helix-leucine zipper (bHLH-ZIP) transcription factors [11]. SREBPs directly activate the expression of numerous genes linked to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. The mammalian genome encodes three SREBPs, designated SREBP-1a, SREBP-1c, and SREBP-2 [33]. In comparison to SREBP-1c, SREBP-1a has a longer transcription activation domain at its N-terminus which is capable of recruiting co-activators for transcription [33]. As such, SREBP-1a is a more potent activator of all SREBP-responsive genes for fatty acid and cholesterol synthesis, whereas SREBP-1c can only activate fatty acid synthesis [12,29]. SREBPs are synthesized as inactive precursors and the N-terminal portion, released from the SREBP precursor by proteolysis, enters the nucleus and becomes active transcriptional factors [3]. Nuclear SREBPs activate transcription by binding to SRE sequence in the promoter regions of target genes [11].

Hepatitis B virus (HBV) infection is a global health problem with 350–400 million people being chronic carriers [2]. HBV has a partially double-stranded circular DNA genome coding for core, surface, polymerase, and the X (HBx) proteins [6]. Two viral enhancers promote HBV transcription [23]. Enhancer II/core promoter sequence regulates the transcription of 3.5-kb pregenomic RNA, a key step in HBV replication cycle. HBx increases HBV replication and activates HBV enhancer II/core promoter [5,15,21]. HBx also modulates host cellular functions including lipid metabolism [25,32]. In this study, we investigated activation of SREBP-1a by HBx and its involvement in regulating HBV enhancer II/core promoter by HBx.

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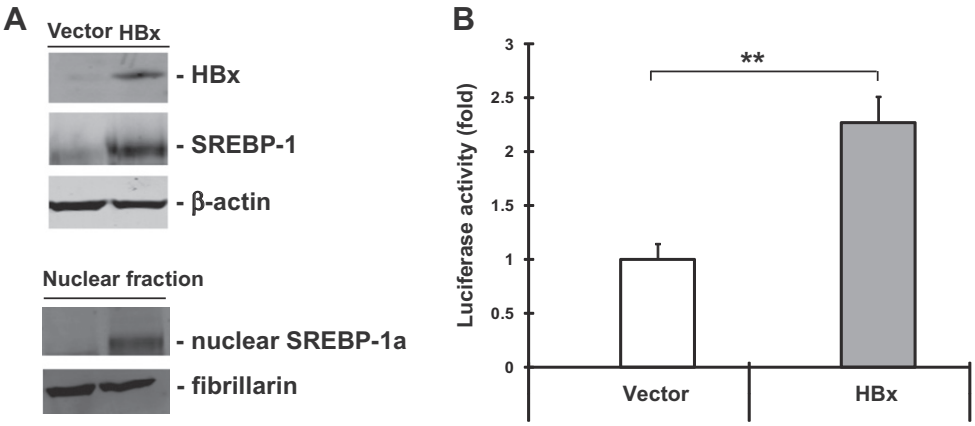

Fig. 1. Expression of HBx increases the level of nuclear SREBP-1a and its transactivation activity. (A) In the top panel, the levels of HBx, SREBP-1, and β-actin in Huh-7 cells after transfection with an HBx-expressing plasmid or the vector were analyzed by immunoblotting. In the bottom panel, levels of SREBP-1a in the nuclear fraction were analyzed by immunoblotting after co-transfection with plasmids expressing Flag-SREBP-1a and HBx or vector. The blots were probed with antibodies against Flag-tag or fibrillarin. (B) A luciferase reporter driven by SRE sequences was co-transfected with a plasmid encoding Flag-SREBP-1a together with HBx-expressing plasmid or vector control into Huh-7 cells. Luciferase assay was performed using the cell lysates. Luciferase activity was expressed as fold change relative to vector control. The statistical difference between samples was demonstrated as ** if $p \leqslant 0.01$.

2. Materials and methods

2.1. Plasmids and antibodies

The coding sequence of HBx was amplified by PCR from plasmids pRBK HBx or pawy1.2 [8,28] and cloned in-frame with the myc tag into the pEF/cyto/myc vector (Invitrogen). HBV enhancer II/core promoter sequence [27] was cloned into pGL4.14 vector (Promega), generating pGL4-HBV EN2/CP where the expression of luciferase gene was controlled by HBV enhancer II/core promoter. Flag-tagged SREBP-1a (aa. 1–517) was amplified from an SREBP-1a plasmid [30] and inserted into the pCMV2 Flag vector (Sigma–Aldrich) [14]. Plasmid pSRE-Luc containing three copies of SRE sequences was provided by Shimano [1]. Human SREBP-1a promoter – luciferase reporters containing different lengths of the SREBP-1a promoter were described previously [10]. Mutant SREBP-1a promoters with mutations for the binding sequences for C/EBP (CCAAT/enhancer binding protein) and E4BP4 (Adenovirus E4 promoter binding protein 4) were generated by site-directed mutagenesis and confirmed by DNA sequencing (Fig. 3). Plasmids expressing C/EBPα [4] and E4BP4 (Open Biosystems) were used. SREBP-1-targeting microRNA (miRNA) with target sequence of 5′ CCTGGTCTACCATAAGCTGCA 3′ was constructed in pcDNA6.2-GW/EmGFP miR vector (Invitrogen).

SREBP-1, Flag (M2), fibrillarin, β-actin and Myc epitope antibodies were from Santa Cruz Biotechnology, Sigma–Aldrich, and Cell Signaling Technology, respectively. Anti-HBx antibody was provided by Richardson [8].

2.2. Cell culture, transfection and nuclear fractionation

Huh-7 cells [20] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Huh-7 cells were transfected using the calcium phosphate precipitation method as previously described [13]. Nuclear fractions were isolated as described [36].

2.3. Immunoblotting analysis

Huh-7 cells were collected with a Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Immunoblotting was performed as described [14,36]. For SREBP experiments, cells were treated with a protease inhibitor ALLN (25 μg/ml, Calbiochem) for 1 h prior to lysis.

2.4. Reverse transcription and real-time PCR

RNA was isolated from Huh-7 cells with Trizol (Invitrogen) followed by DNase I (Invitrogen) digestion. Reverse transcription was carried out by Superscript II (Invitrogen) and random priming. Real-time PCR was performed with primers SREBP-1a-FD (5′ CGCTGCTGACCGACAT 3′) and SREBP-1a-rev (5′ CAAGAGAGGAGCTCAATG 3′) using SYBR Green based detection system. Housekeeping gene GUSB was amplified in parallel by primers GUSB-FD (5′ GGTGCTGAGGATTGGCAGTG 3′) and GUSB-rev (5′ CGCACTTCCAACTTGAACAGG 3′). Data was analyzed by Bio-Rad iQ5 program.

2.5. Luciferase assay

Huh-7 cells were lysed in a Passive Lysis Buffer (Promega) and luciferase activity was determined using luciferase assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Results were analyzed for statistical differences using Student t test. A p value of $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. HBx increases the level of SREBP-1a in the nucleus

HBx coding sequence was cloned into the pEF-cyto-myc vector in-frame with the myc tag. The resulting plasmid was transfected into Huh-7 cells and the cell lysates were analyzed in immunoblotting. As shown in Fig. 1A, a specific protein band was recognized by an HBx-specific antibody and a myc-tag antibody (not shown) in cells transfected with HBx-expressing plasmid, but not in vector-transfected cells. The level of β-actin was used as loading control. These results demonstrated the expression of HBx protein after transfection.

Given the importance of SREBP-1a in modulating lipid metabolism, we explored the role of HBx in SREBP-1a activation. Huh-7 cells were transfected with HBx-expressing plasmid and vector control. The level of SREBP-1 was analyzed by immunoblotting using an anti-SREBP-1 antibody. As shown in the upper panel of Fig. 1A, expression of HBx was associated with increased level of SREBP-1 compared to control. Because we were interested in SREBP-1a levels especially in the nucleus as the active form, however the SREBP-1 antibody cannot distinguish SREBP-1a from another isoform SREBP-1c. Therefore we used a plasmid expressing

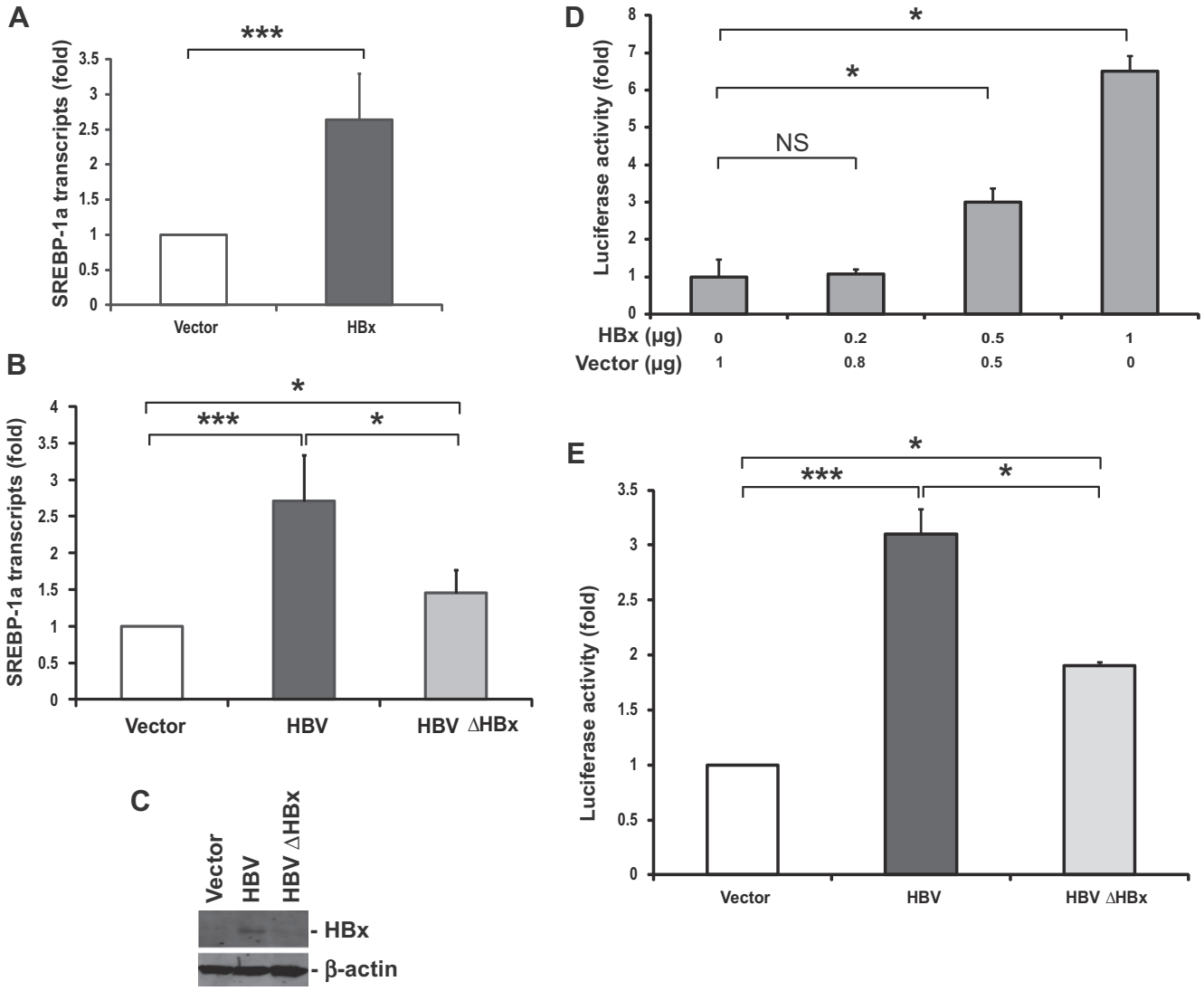

Fig. 2. HBx up-regulates SREBP-1a transcription. (A and B) Huh-7 cells were transfected with vector or HBx-expressing plasmids (A), vector, HBV, or HBV ΔHBx (B). The levels of SREBP-1a transcript were analyzed by reverse-transcription real-time PCR. (C) The protein levels of HBx and β-actin in Huh-7 cells after transfection with vector, HBV, or HBV ΔHBx were determined by immunoblotting. (D and E) Huh-7 cells were co-transfected with a human SREBP-1a promoter (−1008/+194)-luciferase reporter plasmid with increasing amounts of HBx-expressing plasmid (D) or vector, HBV, or HBV ΔHBx (E). Luciferase activities were expressed as fold changes relative to vector control. The statistical differences between samples were demonstrated as NS for not significant, * if $p \leqslant 0.05$, or *** if $p \leqslant 0.001$.

Flag-tagged SREBP-1a and an anti-Flag antibody to examine nuclear SREBP-1a levels (lower panel of Fig. 1A). The results showed that the nuclear SREBP-1a protein level was increased in HBx transfected cells than in vector transfected cells. To determine whether the increased SREBP-1a correlates with its enhanced activity as a transcription factor, we used an SRE-luciferase reporter where the luciferase expression is directly controlled by three copies of SRE sequences (ATCACCCCAC, pSRE-Luc) [1]. This is because SREBP-1a activates transcription of its target genes by binding to the SRE sequence. As shown in Fig. 1B, HBx expression significantly increased SRE-driven luciferase activity by SREBP-1a compared to control. These results indicate that expression of HBx increases SREBP-1a level in the nucleus and its transcription factor activity.

3.2. HBx activates SREBP-1a transcription

SREBPs are often regulated at the transcription level. To determine whether HBx regulates SREBP-1a transcription, we measured the level of SREBP-1a transcript by real-time PCR after HBx expression in Huh-7 cells. As shown in Fig. 2A, HBx expression resulted in more than 2-fold increase in SREBP-1a transcript level in comparison to control. A greater than one genome length HBV plasmid is a widely used model for HBV. As such, we transfected HBV wild-type genome (pawy1.2) and HBV without HBx (pawy1.2∗7) [28] into Huh-7 cells and determined SREBP-1a transcript levels. As shown in Fig. 2B, SREBP-1a transcript level in HBV-transfected cells were significantly higher than those in vector- or HBV ΔHBx-transfected cells. The expression of HBx was confirmed after HBV plasmid transfection, whereas no HBx could be detected in vector- or HBVΔHBx-transfected cells (Fig. 2C). These results indicate that HBx up-regulates SREBP-1a transcription.

To further characterize SREBP-1a transcription up-regulation by HBx, Huh-7 cells were co-transfected with a luciferase reporter under the control of human SREBP-1a promoter (−1008) [10] and increasing amounts of HBx-expressing plasmid. The total amounts of plasmid DNA used for transfection were kept constant by adding appropriate amounts of the vector plasmid. Luciferase assay showed that HBx significantly activated SREBP-1a promoter activity in a dose-dependent manner in comparison to vector control (Fig. 2D). Similarly, HBV plasmid transfection resulted in

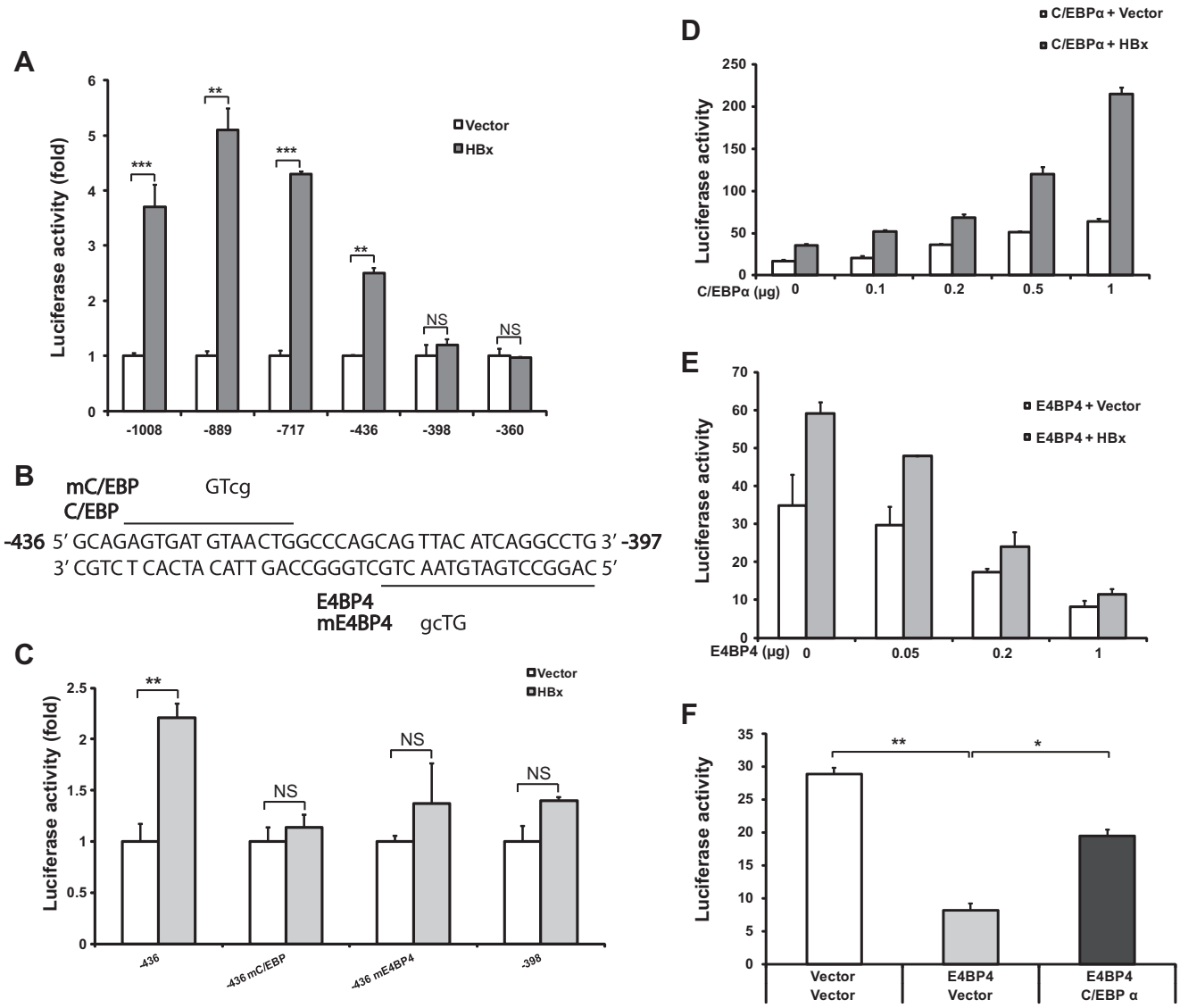

Fig. 3. The roles of transcription factors C/EBP and E4BP4 in SREBP-1a promoter regulation by HBx. (A) Huh-7 cells were transfected with SREBP-1a promoters of different lengths together with HBx-expressing plasmid or vector control. SREBP-1a promoter activity was determined by luciferase assay. (B) SREBP-1a promoter sequence −436 to −397. The binding motifs as well as the mutated sequences for C/EBP and E4BP4 are shown. (C) Sequence integrity of the SREBP-1a promoter −436 to −398 region is required for its activation by HBx. Huh-7 cells were co-transfected with wild-type or mutant SREBP-1a promoters with HBx-expressing plasmid or vector control. SREBP-1a promoter activity was determined by luciferase assay. (D and E) Huh-7 cells were co-transfected with SREBP-1a-promoter luciferase reporter (−436/+194), HBx-expressing plasmid or vector control, together with increasing amounts of C/EBPα (D) or E4BP4 (E). SREBP-1a promoter activity was determined by luciferase assay. (F) Huh-7 cells were co-transfected with SREBP-1a-promoter luciferase reporter (−436/+194), HBx-expressing plasmid, together with vector, E4BP4-expressing plasmid, and C/EBPα-expressing plasmid. SREBP-1a promoter activity was determined by luciferase assay. The statistical differences between samples were demonstrated as NS for not significant, * if $p \leqslant 0.05$, ** if $p \leqslant 0.01$, or *** if $p \leqslant 0.001$.

significantly higher luciferase activity than vector- or HBVΔHBx-transfection (Fig. 2E).

Next, we wanted to map the regions on the SREBP-1a promoter that were required for its activation by HBx. We used five truncated SREBP-1a promoters −889, −717, −436, −398, and −360 (Fig. 3A). Luciferase assay results showed that deletion from −1008 to −436 in the SREBP-1a promoter did not affect its activation by HBx, whereas the activation was abolished when the −398 and −360 promoters were used. These results indicate that the sequence between −436 and −398 in the SREBP-1a promoter is required for its activation by HBx.

3.3. Effects of C/EBP and E4BP4 on SREBP-1a regulation by HBx

Sequence analysis by the MatINSPECTOR prediction program indicated that the −436 to −398 region in the SREBP-1a promoter contains binding motifs for transcription factors C/EBP and E4BP4 (Fig. 3B). To gain the first insights into the role of these two factors in SREBP-1a regulation by HBx, we mutated the binding sequences for each of the factors in the SREBP-1a promoter (Fig. 3C). These mutant SREBP-1a promoters were used in co-transfection experiments to test their activity after HBx expression. As shown in Fig. 3C, HBx failed to activate these mutant SREBP-1a promoters. These results suggested that the integrity of C/EBP and E4BP4 binding sequences is necessary for SREBP-1a regulation by HBx. Previous research has established that C/EBP and E4BP4 have divergent effects on transcription, although both belong to a family of basic leucine zipper (bZIP) proteins [7,34].

To determine the effects of these transcription factors in SREBP-1a regulation by HBx, we studied SREBP-1a promoter activity after ectopic expression of C/EBPα or E4BP4. There are six isoforms in the C/EBP family and C/EBPα was isolated from the liver [31,34].

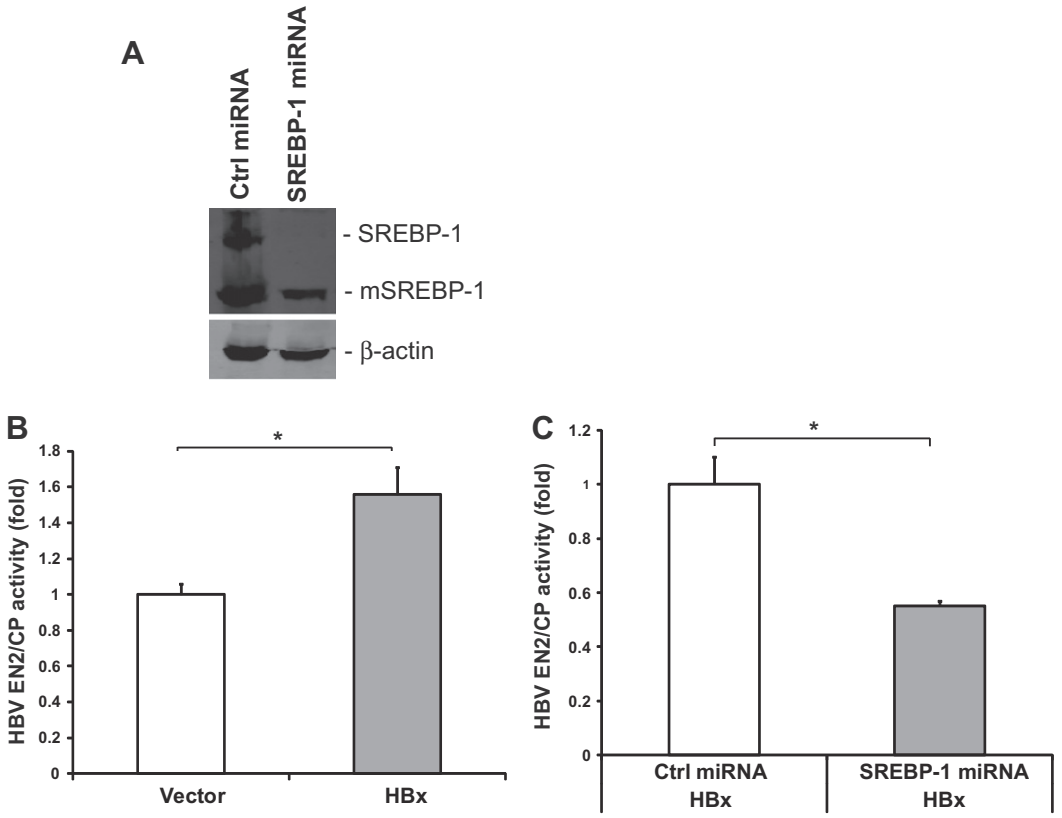

Fig. 4. The role of SREBP-1 in HBV enhancer II/core promoter activation by HBx. (A) Huh-7 cells were transfected with control or SREBP-1 miRNAs. The levels of precursor and mature SREBP-1 were determined by immunoblotting. (B) Huh-7 cells were co-transfected with HBV enhancer II/core promoter-luciferase reporter pGL4 HBV EN2/CP and vector or HBx-expressing plasmids. Luciferase activities after HBx expression were expressed as fold changes relative to vector control. (C) Huh-7 cells were co-transfected with pGL4 HBV EN2/CP, HBx-expressing plasmid, and control or SREBP-1 miRNA. Luciferase activities after SREBP-1 knockdown were expressed as fold changes relative to control miRNA-transfection. The statistical differences between samples were demonstrated as * if $p \leqslant 0.05$.

Therefore, we used a plasmid expressing C/EBPα. As shown in Fig. 3D, transfection with increasing amounts of C/EBPα resulted in dose-dependent SREBP-1a promoter activation in both vector and HBx expressing cells. In contrast, increasing amounts of E4BP4 resulted in dose-dependent decrease in SREBP-1a promoter activity in both vector and HBx expressing cells (Fig. 3E). These results indicated that C/EBPα is as an activator, whereas E4BP4 is a repressor for SREBP-1a promoter regulation by HBx.

Opposing effects of C/EBP and E4BP4 on SREBP-1a promoter regulation by HBx raised a question as to whether the inhibitory effect of E4BP4 can be overcome by C/EBP. To answer this question, Huh-7 cells were co-transfected with HBx-expressing plasmid and SREBP-1a promoter (−436) – luciferase reporter, together with E4BP4 alone or with C/EBPα. Corresponding vectors were used as controls. As shown in Fig. 3F, the inhibition of SREBP-1a promoter activity by E4BP4 was significantly reversed upon ectopic expression of C/EBPα E4BP4. These results suggest that C/EBPα has a dominantly activating effect on SREBP-1a promoter activity.

3.4. Effect of SREBP-1 in HBV enhancer II/core promoter activation by HBx

Our results so far have shown that HBx activates SREBP-1a. Previous studies demonstrated that HBx increases HBV enhancer II/core promoter activity [5,15]. We therefore interested in determining whether SREBP-1a is involved in this process. For this purpose, we used a miRNA to knockdown SREBP-1 expression in Huh-7 cells. As shown in Fig. 4A, the levels of both precursor and mature SREBP-1 proteins were reduced by SREBP-1 miRNA in comparison to control miRNA. When Huh-7 cells were transfected with vector or HBx-expressing plasmids together with pGL4-HBV EN2/CP, modest but significant 1.5-fold transactivation of enhancer II/core promoter by HBx was observed (Fig. 4B). This result is consistent with other studies showing activation of enhancer II/core promoter by HBx [5,15]. Upon knockdown SREBP-1, HBx was no longer able to transactivate enhancer II/core promoter (Fig. 4C). These results demonstrate that SREBP-1 is involved in HBV enhancer II/core promoter transactivation by HBx.

4. Discussion

HBx is a multifunctional protein. Here, we demonstrated that HBx activates SREBP-1a, a transcription factor involved in several (patho)-physiological conditions such as lipogenesis and carcinogenesis [18,19]. We further demonstrated that SREBP-1 is involved in HBV enhancer II/core promoter activation by HBx.

To become an active transcription factor, SREBP-1a needs to enter the nucleus and therefore nuclear SREBP-1a level reflects its activity. As such, we first demonstrated that HBx expression is associated with increased level of SREBP-1a in the nucleus (Fig. 1A). An SRE-driven luciferase reporter assay confirmed that SREBP-1a in HBx-transfected cells is indeed transcriptionally active (Fig. 1B).

The expression of SREBP-1a can be regulated at the transcription level [10]. Our results demonstrated that HBx can significantly up-regulate the activity of SREBP-1a promoter in a dose-dependent manner (Fig. 2D). More importantly, we showed that HBx in the context of HBV can also transactivate SREBP-1a transcription using a plasmid-based HBV model (Fig. 2B and E). Mapping the sequences in the SREBP-1a promoter required for activation by HBx led us to concentrate on two bZIP transcription factors, namely C/EBP and E4BP4. Mutating the C/EBP binding sequence on the

SREBP-1a promoter canceled SREBP-1a promoter activation by HBx (Fig. 3C). Consistently, ectopic expression of C/EBPα demonstrated that it can further enhance SREBP-1a promoter activation by HBx (Fig. 3D). These results suggest that C/EBP is required for SREBP-1a activation by HBx.

Ectopic expression of E4BP4, on the other hand, has an opposing, inhibiting effect on SREBP-1a promoter activity (Fig. 3E). This finding is consistent with the established repressor activity of E4BP4 on transcription [7]. However, when the E4BP4 binding sequence was mutated on the SREBP-1a promoter, we did not see the expected increase of the promoter activity (Fig. 3C). The underlying reason is not clear. Several possible mechanisms exist. The binding sequences on gene promoters for bZIP transcription factors are similar, which may result in DNA binding competition by these factors [7]. In fact, it has been shown that C/EBP and E4BP4 can compete with each other in DNA binding and regulate transcription in a competitive manner [22,26]. Therefore, we cannot exclude the possibility that C/EBP can also bind the E4BP4 sequence on SREBP-1a promoter and positively regulate its activity. As such, elimination of E4BP4 binding sequence can potentially affect the activities of both E4BP4 and C/EBP. Another possibility is that mutating the E4BP4 binding motif might somehow negatively affect the binding of C/EBP to its recognition sequence on the SREBP-1a promoter because they are in a very close proximity. In addition, b-ZIP factors regulate transcription by forming homo- and/or hetero-dimers [9]. According to a molecular interaction model, C/EBP and E4BP4 may interact with each other due to the presence of an asparagine residue in the "a" position of their bZIP domains which would favor heterodimerization [35]. It is reasonable to assume that elimination of E4BP4 binding sequence on the SREBP-1a promoter may increase the amount of free E4BP4. Then it is possible that more E4BP4 proteins can interact with C/EBP and interfere with the activity of C/EBP, resulting in reduced SREBP-1a promoter activation. If this is true, one would expect that increasing the amount of C/EBP should increase SREBP-1a promoter activity in the presence of E4BP4. This has been actually observed in our experiments (Fig. 3F). However, the exact mechanisms warrant further investigation.

Recent studies have shown that HBx can activate SREBP-1c, the other isoform of SREBP-1, through liver X receptor [16,17,24]. Adding to these previous findings, our study has demonstrated that HBx can also activate SREBP-1a through a different and complex mechanism involving at least two transcription factors, C/EBP and E4BP4. More importantly, SREBP-1a is a more potent transcription activator for both fatty acid and cholesterol synthesis pathways [12,29,33]. In contrast, SREBP-1c is less active and only activates fatty acid synthesis. The functional significance of SREBP-1 activation in HBV biology and pathogenesis has not been characterized. Towards this goal, we showed that knocking down SREBP-1 abolishes activation of HBV enhancer II/core promoter activation by HBx (Fig. 4). Since the miRNA sequence we used does not distinguish between SREBP-1a and -1c, further experiments are needed to determine isoform-specific effects of SREBP-1.

In conclusion, our results demonstrated that HBx activates the SREBP-1a activity by a complex mechanism involving two bZIP transcription factors C/EBP and E4BP4 with the former being the dominant factor leading to SREBP-1a promoter activation. Furthermore, we showed that SREBP-1 is involved in HBV enhancer II/core promoter activation by HBx.

Acknowledgments

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