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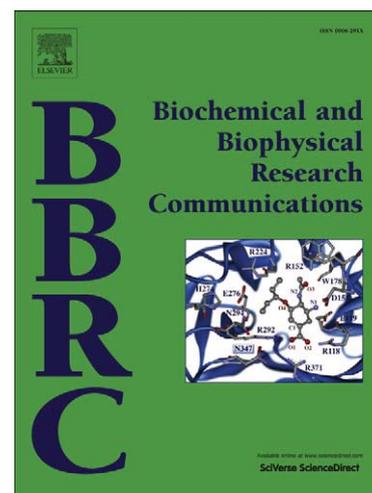
1   **SREBP-1a activation by HBx and the effect on hepatitis B virus enhancer II/core promoter**

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10   Running title: HBx activates HBV enhancer II/core promoter through SREBP-1

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28   **Abstract**

29   Hepatitis B virus (HBV) X protein (HBx) plays an important role in HBV pathogenesis by

30   regulating gene expression. Sterol regulatory element binding protein-1a (SREBP-1a) is a key

31   transcriptional factor for modulating fatty acid and cholesterol synthesis. Here we demonstrated that HBx

32   increased mature SREBP-1a protein level in the nucleus and its activity as a transcription factor. We

33   further showed that the up-regulation of SREBP-1a by HBx occurred at the transcriptional level after

34   ectopic expression and in the context of HBV replication. Deletional analysis using SREBP-1a promoter

35   revealed that the sequence from -436 to -398 in the promoter was required for its activation by HBx. This

36   promoter region possesses the binding sequences for two basic leucine zipper (b-ZIP) transcription

37   factors, namely C/EBP and E4BP4. Mutagenesis of the binding sequences on the SREBP-1a promoter and

38   ectopic expression experiments demonstrated that C/EBPα enhanced SREBP-1a activation by HBx, while

39   E4BP4 had an inhibitory effect. C/EBPα was able to significantly reverse the inhibitory activity of E4BP4

40   on SREBP-1a promoter. These results demonstrated that HBx activates SREBP-1a activity at the

41   transcription level through a complex mechanism involving two bZIP transcription factors C/EBP and

42   E4BP4 with C/EBP being the dominant positive factor. Finally, we showed that knocking down SREBP-1

43   abolishes HBV enhancer II/core promoter activation by HBx.

44

45   **Keywords**: HBx; SREBP-1a; C/EBP; E4BP4; HBV enhancer II/core promoter; transcription
46

[Type text]

47 **1. Introduction**

48   Sterol regulatory element-binding proteins (SREBPs) belong to the family of basic-helix-loop-helix-

49 leucine zipper (bHLH-ZIP) transcription factors [11]. SREBPs directly activate the expression of

50 numerous genes linked to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and

51 phospholipids. The mammalian genome encodes three SREBPs, designated SREBP-1a, SREBP-1c, and

52 SREBP-2 [33]. In comparison to SREBP-1c, SREBP-1a has a longer transcription activation domain at its

53 N-terminus which is capable of recruiting co-activators for transcription [33]. As such, SREBP-1a is a

54 more potent activator of all SREBP-responsive genes for fatty acid and cholesterol synthesis, whereas

55 SREBP-1c can only activate fatty acid synthesis [12;29]. SREBPs are synthesized as inactive precursors

56 and the N-terminal portion, released from the SREBP precursor by proteolysis, enters the nucleus and

57 becomes active transcriptional factors [3]. Nuclear SREBPs activate transcription by binding to SRE

58 sequence in the promoter regions of target genes [11].

59   Hepatitis B virus (HBV) infection is a global health problem with 350 to 400 million people being

60 chronic carriers [2]. HBV has a partially double-stranded circular DNA genome coding for core, surface,

61 polymerase, and the X (HBx) proteins [6]. Two viral enhancers promote HBV transcription [23].

62 Enhancer II/core promoter sequence regulates the transcription of 3.5-kb pregenomic RNA, a key step in

63 HBV replication cycle. HBx increases HBV replication and activates HBV enhancer II/core promoter

64 [5;15;21]. HBx also modulates host cellular functions including lipid metabolism [25;32]. In this study,

65 we investigated activation of SREBP-1a by HBx and its involvement in regulating HBV enhancer II/core

66 promoter by HBx.

67

68 **2. Materials and Methods**

69 *2.1. Plasmids and antibodies*

70   The coding sequence of HBx was amplified by PCR from plasmids pRBK HBx or pawy1.2 [8;28] and

71 cloned in-frame with the myc tag into the pEF/cyto/myc vector (Invitrogen). HBV enhancer II/core

72 promoter sequence [27] was cloned into pGL4.14 vector (Promega), generating pGL4-HBV EN2/CP

[Type text]

73   where the expression of luciferase gene was controlled by HBV enhancer II/core promoter. Flag-tagged

74   SREBP-1a (aa. 1-517) was amplified from an SREBP-1a plasmid [30] and inserted into the pCMV2 Flag

75   vector (Sigma-Aldrich) [14]. Plasmid pSRE-Luc containing three copies of SRE sequences was provided

76   by Dr. Shimano [1]. Human SREBP-1a promoter - luciferase reporters containing different lengths of the

77   SREBP-1a promoter were described previously [10]. Mutant SREBP-1a promoters with mutations for the

78   binding sequences for C/EBP (CCAAT/enhancer binding protein) and E4BP4 (Adenovirus E4 promoter

79   binding protein 4) were generated by site-directed mutagenesis and confirmed by DNA sequencing

80   (Figure 3). Plasmids expressing C/EBPα [4] and E4BP4 (Open Biosystems) were used. SREBP-1-

81   targeting microRNA (miRNA) with target sequence of 5' CCTGGTCTACCATAAGCTGCA 3' was

82   constructed in pcDNA6.2-GW/EmGFP miR vector (Invitrogen).

83   SREBP-1, Flag (M2), fibrillarin, β-actin and Myc epitope antibodies were from Santa Cruz

84   Biotechnology, Sigma-Aldrich, and Cell Signaling Technology, respectively. Anti-HBx antibody was

85   provided by Dr. Richardson [8].

86

87   *2.2. Cell culture, transfection and nuclear fractionation*

88   Huh-7 cells [20] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v)

89   fetal bovine serum (FBS). Huh-7 cells were transfected using the calcium phosphate precipitation method

90   as previously described [13]. Nuclear fractions were isolated as described [36].

91

92   *2.3. Immunoblotting analysis*

93   Huh-7 cells were collected with a Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM

94   phenylmethylsulphonyl fluoride (PMSF). Immunoblotting was performed as described [14;36]. For

95   SREBP experiments, cells were treated with a protease inhibitor ALLN (25 µg/ml, Calbiochem) for 1

96   hour prior to lysis.

97

[Type text]

98   *2.4. Reverse transcription and real-time PCR*

99   RNA was isolated from Huh-7 cells with Trizol (Invitrogen) followed by DNase I (Invitrogen)

100   digestion. Reverse transcription was carried out by Superscript II (Invitrogen) and random priming. Real-

101   time PCR was performed with primers SREBP-1a-FD (5' CGCTGCTGACCGACAT 3') and SREBP-1a-

102   rev (5' CAAGAGAGGAGCTCAATG 3') using SYBR Green based detection system. Housekeeping

103   gene GUSB was amplified in parallel by primers GUSB-FD (5' GGTGCTGAGGATTGGCAGTG 3') and

104   GUSB-rev (5' CGCACTTCCAACTTGAACAGG 3'). Data was analyzed by Bio-Rad iQ5 program.

105

106   *2.5. Luciferase assay*

107   Huh-7 cells were lysed in a Passive Lysis Buffer (Promega) and luciferase activity was determined

108   using luciferase assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Results were

109   analyzed for statistical differences using Student t test. A p value of ≤ 0.05 was considered statistically

110   significant.

111

112   **3.   Results**

113   *3.1. HBx increases the level of SREBP-1a in the nucleus*

114   HBx coding sequence was cloned into the pEF-cyto-myc vector in-frame with the myc tag. The

115   resulting plasmid was transfected into Huh-7 cells and the cell lysates were analyzed in immunoblotting.

116   As shown in Fig. 1A, a specific protein band was recognized by an HBx-specific antibody and a myc-tag

117   antibody (not shown) in cells transfected with HBx-expressing plasmid, but not in vector-transfected cells.

118   The level of β-actin was used as loading control. These results demonstrated the expression of HBx

119   protein after transfection.

120   Given the importance of SREBP-1a in modulating lipid metabolism, we explored the role of HBx in

121   SREBP-1a activation. Huh-7 cells were transfected with HBx-expressing plasmid and vector control. The

122   level of SREBP-1 was analyzed by immunoblotting using an anti-SREBP-1 antibody. As shown in the

[Type text]

123 upper panel of Fig. 1A, expression of HBx was associated with increased level of SREBP-1 compared to

124 control. Because we were interested in SREBP-1a levels especially in the nucleus as the active form,

125 however the SREBP-1 antibody cannot distinguish SREBP-1a from another isoform SREBP-1c.

126 Therefore we used a plasmid expressing Flag-tagged SREBP-1a and an anti-Flag antibody to examine

127 nuclear SREBP-1a levels (lower panel of Fig. 1A). The results showed that the nuclear SREBP-1a protein

128 level was increased in HBx transfected cells than in vector transfected cells. To determine whether the

129 increased SREBP-1a correlates with its enhanced activity as a transcription factor, we used an SRE-

130 luciferase reporter where the luciferase expression is directly controlled by three copies of SRE sequences

131 (ATCACCCCAC, pSRE-Luc) [1]. This is because SREBP-1a activates transcription of its target genes by

132 binding to the SRE sequence. As shown in Fig. 1B, HBx expression significantly increased SRE-driven

133 luciferase activity by SREBP-1a compared to control. These results indicate that expression of HBx

134 increases SREBP-1a level in the nucleus and its transcription factor activity.

135

136 *3.2. HBx activates SREBP-1a transcription*

137 SREBPs are often regulated at the transcription level. To determine whether HBx regulates SREBP-1a

138 transcription, we measured the level of SREBP-1a transcript by real-time PCR after HBx expression in

139 Huh-7 cells. As shown in Fig. 2A, HBx expression resulted in more than 2-fold increase in SREBP-1a

140 transcript level in comparison to control. A greater than one genome length HBV plasmid is a widely used

141 model for HBV. As such, we transfected HBV wild-type genome (pawy1.2) and HBV without HBx

142 (pawy1.2*7) [28] into Huh-7 cells and determined SREBP-1a transcript levels. As shown in Fig. 2B,

143 SREBP-1a transcript level in HBV-transfected cells were significantly higher than those in vector- or

144 HBV ΔHBx-transfected cells. The expression of HBx was confirmed after HBV plasmid transfection,

145 whereas no HBx could be detected in vector- or HBVΔHBx-transfected cells (Fig. 2C). These results

146 indicate that HBx up-regulates SREBP-1a transcription.

147 To further characterize SREBP-1a transcription up-regulation by HBx, Huh-7 cells were co-

148 transfected with a luciferase reporter under the control of human SREBP-1a promoter (-1008) [10] and

[Type text]

149 increasing amounts of HBx-expressing plasmid. The total amounts of plasmid DNA used for transfection

150 were kept constant by adding appropriate amounts of the vector plasmid. Luciferase assay showed that

151 HBx significantly activated SREBP-1a promoter activity in a dose-dependent manner in comparison to

152 vector control (Fig. 2D). Similarly, HBV plasmid transfection resulted in significantly higher luciferase

153 activity than vector- or HBVΔHBx-transfection (Fig. 2E).

154   Next, we wanted to map the regions on the SREBP-1a promoter that were required for its activation

155 by HBx. We used five truncated SREBP-1a promoters -889, -717, -436, -398, and -360 (Fig. 3A).

156 Luciferase assay results showed that deletion from -1008 to -436 in the SREBP-1a promoter did not affect

157 its activation by HBx, whereas the activation was abolished when the -398 and -360 promoters were used.

158 These results indicate that the sequence between -436 and -398 in the SREBP-1a promoter is required for

159 its activation by HBx.

160

161 *3.3. Effects of C/EBP and E4BP4 on SREBP-1a regulation by HBx*

162   Sequence analysis by the MatINSPECTOR prediction program indicated that the -436 to -398 region

163 in the SREBP-1a promoter contains binding motifs for transcription factors C/EBP and E4BP4 (Fig. 3B).

164 To gain the first insights into the role of these two factors in SREBP-1a regulation by HBx, we mutated

165 the binding sequences for each of the factors in the SREBP-1a promoter (Fig. 3C). These mutant SREBP-

166 1a promoters were used in co-transfection experiments to test their activity after HBx expression. As

167 shown in Fig. 3C, HBx failed to activate these mutant SREBP-1a promoters. These results suggested that

168 the integrity of C/EBP and E4BP4 binding sequences is necessary for SREBP-1a regulation by HBx.

169 Previous research has established that C/EBP and E4BP4 have divergent effects on transcription, although

170 both belong to a family of basic leucine zipper (bZIP) proteins [7;34].

171   To determine the effects of these transcription factors in SREBP-1a regulation by HBx, we

172 studied SREBP-1a promoter activity after ectopic expression of C/EBPα or E4BP4. There are six isoforms

173 in the C/EBP family and C/EBPα was isolated from the liver [31;34]. Therefore, we used a plasmid

174 expressing C/EBPα. As shown in Fig. 3D, transfection with increasing amounts of C/EBPα resulted in

[Type text]

175 dose-dependent SREBP-1a promoter activation in both vector and HBx expressing cells. In contrast,

176 increasing amounts of E4BP4 resulted in dose-dependent decrease in SREBP-1a promoter activity in both

177 vector and HBx expressing cells (Figs. 3E). These results indicated that C/EBPα is as an activator,

178 whereas E4BP4 is a repressor for SREBP-1a promoter regulation by HBx.

179 Opposing effects of C/EBP and E4BP4 on SREBP-1a promoter regulation by HBx raised a

180 question as to whether the inhibitory effect of E4BP4 can be overcome by C/EBP. To answer this question,

181 Huh-7 cells were co-transfected with HBx-expressing plasmid and SREBP-1a promoter (-436) - luciferase

182 reporter, together with E4BP4 alone or with C/EBPα. Corresponding vectors were used as controls. As

183 shown in Fig. 3F, the inhibition of SREBP-1a promoter activity by E4BP4 was significantly reversed

184 upon ectopic expression of C/EBPα E4BP4. These results suggest that C/EBPα has a dominantly

185 activating effect on SREBP-1a promoter activity.

186

187 *3.4. Effect of SREBP-1 in HBV enhancer II/core promoter activation by HBx*

188 Our results so far have shown that HBx activates SREBP-1a. Previous studies demonstrated that HBx

189 increases HBV enhancer II/core promoter activity [5;15]. We therefore interested in determining whether

190 SREBP-1a is involved in this process. For this purpose, we used a miRNA to knockdown SREBP-1

191 expression in Huh-7 cells. As shown in Fig. 4A, the levels of both precursor and mature SREBP-1

192 proteins were reduced by SREBP-1 miRNA in comparison to control miRNA. When Huh-7 cells were

193 transfected with vector or HBx-expressing plasmids together with pGL4-HBV EN2/CP, modest but

194 significant 1.5-fold transactivation of enhancer II/core promoter by HBx was observed (Fig. 4B). This

195 result is consistent with other studies showing activation of enhancer II/core promoter by HBx [5;15].

196 Upon knockdown SREBP-1, HBx was no longer able to transactivate enhancer II/core promoter (Fig. 4C).

197 These results demonstrate that SREBP-1 is involved in HBV enhancer II/core promoter transactivation by

198 HBx.

199

200 **4. Discussion**

[Type text]

201   HBx is a multifunctional protein. Here, we demonstrated that HBx activates SREBP-1a, a

202   transcription factor involved in several (patho)-physiological conditions such as lipogenesis and

203   carcinogenesis [18;19]. We further demonstrated that SREBP-1 is involved in HBV enhancer II/core

204   promoter activation by HBx.

205   To become an active transcription factor, SREBP-1a needs to enter the nucleus and therefore nuclear

206   SREBP-1a level reflects its activity. As such, we first demonstrated that HBx expression is associated

207   with increased level of SREBP-1a in the nucleus (Fig. 1A). An SRE-driven luciferase reporter assay

208   confirmed that SREBP-1a in HBx-transfected cells is indeed transcriptionally active (Fig. 1B).

209   The expression of SREBP-1a can be regulated at the transcription level [10]. Our results demonstrated

210   that HBx can significantly up-regulate the activity of SREBP-1a promoter in a dose-dependent manner

211   (Fig. 2D). More importantly, we showed that HBx in the context of HBV can also transactivate SREBP-1a

212   transcription using a plasmid-based HBV model (Figs. 2B and 2E). Mapping the sequences in the SREBP-

213   1a promoter required for activation by HBx led us to concentrate on two bZIP transcription factors,

214   namely C/EBP and E4BP4. Mutating the C/EBP binding sequence on the SREBP-1a promoter canceled

215   SREBP-1a promoter activation by HBx (Fig. 3C). Consistently, ectopic expression of C/EBPα

216   demonstrated that it can further enhance SREBP-1a promoter activation by HBx (Fig. 3D). These results

217   suggest that C/EBP is required for SREBP-1a activation by HBx.

218   Ectopic expression of E4BP4, on the other hand, has an opposing, inhibiting effect on SREBP-1a

219   promoter activity (Fig. 3E). This finding is consistent with the established repressor activity of E4BP4 on

220   transcription [7]. However, when the E4BP4 binding sequence was mutated on the SREBP-1a promoter,

221   we did not see the expected increase of the promoter activity (Fig. 3C). The underlying reason is not clear.

222   Several possible mechanisms exist. The binding sequences on gene promoters for bZIP transcription

223   factors are similar, which may result in DNA binding competition by these factors [7]. In fact, it has been

224   shown that C/EBP and E4BP4 can compete with each other in DNA binding and regulate transcription in

225   a competitive manner [22;26]. Therefore, we cannot exclude the possibility that C/EBP can also bind the

226   E4BP4 sequence on SREBP-1a promoter and positively regulate its activity. As such, elimination of

[Type text]

227   E4BP4 binding sequence can potentially affect the activities of both E4BP4 and C/EBP. Another

228   possibility is that mutating the E4BP4 binding motif might somehow negatively affect the binding of

229   C/EBP to its recognition sequence on the SREBP-1a promoter because they are in a very close proximity.

230   In addition, b-ZIP factors regulate transcription by forming homo- and/or hetero-dimers [9]. According to

231   a molecular interaction model, C/EBP and E4BP4 may interact with each other due to the presence of an

232   asparagine residue in the "a" position of their bZIP domains which would favor heterodimerization [35]. It

233   is reasonable to assume that elimination of E4BP4 binding sequence on the SREBP-1a promoter may

234   increase the amount of free E4BP4. Then it is possible that more E4BP4 proteins can interact with C/EBP

235   and interfere with the activity of C/EBP, resulting in reduced SREBP-1a promoter activation. If this is true,

236   one would expect that increasing the amount of C/EBP should increase SREBP-1a promoter activity in the

237   presence of E4BP4. This has been actually observed in our experiments (Fig. 3F). However, the exact

238   mechanisms warrant further investigation.

239   Recent studies have shown that HBx can activate SREBP-1c, the other isoform of SREBP-1, through

240   liver X receptor [16;17;24]. Adding to these previous findings, our study has demonstrated that HBx can

241   also activate SREBP-1a through a different and complex mechanism involving at least two transcription

242   factors, C/EBP and E4BP4. More importantly, SREBP-1a is a more potent transcription activator for both

243   fatty acid and cholesterol synthesis pathways [12;29;33]. In contrast, SREBP-1c is less active and only

244   activates fatty acid synthesis. The functional significance of SREBP-1 activation in HBV biology and

245   pathogenesis has not been characterized. Towards this goal, we showed that knocking down SREBP-1

246   abolishes activation of HBV enhancer II/core promoter activation by HBx (Fig. 4). Since the miRNA

247   sequence we used does not distinguish between SREBP-1a and -1c, further experiments are needed to

248   determine isoform-specific effects of SREBP-1.

249   In conclusion, our results demonstrated that HBx activates the SREBP-1a activity by a complex

250   mechanism involving two bZIP transcription factors C/EBP and E4BP4 with the former being the

251   dominant factor leading to SREBP-1a promoter activation. Furthermore, we showed that SREBP-1 is

252   involved in HBV enhancer II/core promoter activation by HBx.

     [Type text]

253

260

Figure Legends

262

Figure 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 $p \leq 0.01$.

273

Figure 2. HBx up-regulates SREBP-1a transcription. **(A, 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, E)**. Huh-7 cells were co-transfected with a human SREBP-1a

[Type text]

279　promoter (-1008/+194)-luciferase reporter plasmid with increasing amounts of HBx-expressing

280　plasmid (D) or vector, HBV, or HBV ΔHBx (E). Luciferase activities were expressed as fold

281　changes relative to vector control. The statistical differences between samples were demonstrated

282　as NS for not significant, * if $p \leq 0.05$, or *** if $p \leq 0.001$.

283

284　**Figure 3.** The roles of transcription factors C/EBP and E4BP4 in SREBP-1a promoter regulation

285　by HBx. **(A)**. Huh-7 cells were transfected with SREBP-1a promoters of different lengths

286　together with HBx-expressing plasmid or vector control. SREBP-1a promoter activity was

287　determined by luciferase assay. **(B).** SREBP-1a promoter sequence -436 to -397. The binding

288　motifs as well as the mutated sequences for C/EBP and E4BP4 are shown. **(C).** Sequence

289　integrity of the SREBP-1a promoter -436 to -398 region is required for its activation by HBx.

290　Huh-7 cells were co-transfected with wild-type or mutant SREBP-1a promoters with HBx-

291　expressing plasmid or vector control. SREBP-1a promoter activity was determined by luciferase

292　assay. **(D, E).** Huh-7 cells were co-transfected with SREBP-1a-promoter luciferase reporter (-

293　436/+194), HBx-expressing plasmid or vector control, together with increasing amounts of

294　C/EBPα **(D)** or E4BP4 **(E)**. SREBP-1a promoter activity was determined by luciferase assay. **(F).**

295　Huh-7 cells were co-transfected with SREBP-1a-promoter luciferase reporter (-436/+194), HBx-

296　expressing plasmid, together with vector, E4BP4-expressing plasmid, and C/EBPα-expressing

297　plasmid. SREBP-1a promoter activity was determined by luciferase assay. The statistical

298　differences between samples were demonstrated as NS for not significant, * if $p \leq 0.05$, ** if p

299　≤ 0.01, or *** if $p \leq 0.001$.

300

301　**Figure 4**. The role of SREBP-1 in HBV enhancer II/core promoter activation by HBx. **(A)**. Huh-7

302　cells were transfected with control or SREBP-1 miRNAs. The levels of precursor and mature

303　SREBP-1 were determined by immunoblotting. **(B)**. Huh-7 cells were co-transfected with HBV

304　enhancer II/core promoter-luciferase reporter pGL4 HBV EN2/CP and vector or HBx-expressing

[Type text]

305 plasmids. Luciferase activities after HBx expression were expressed as fold changes relative to

306 vector control. **(C)**. Huh-7 cells were co-transfected with pGL4 HBV EN2/CP, HBx-expressing

307 plasmid, and control or SREBP-1 miRNA. Luciferase activities after SREBP-1 knockdown were

308 expressed as fold changes relative to control miRNA-transfection. The statistical differences

309 between samples were demonstrated as * if $p \leq 0.05$.

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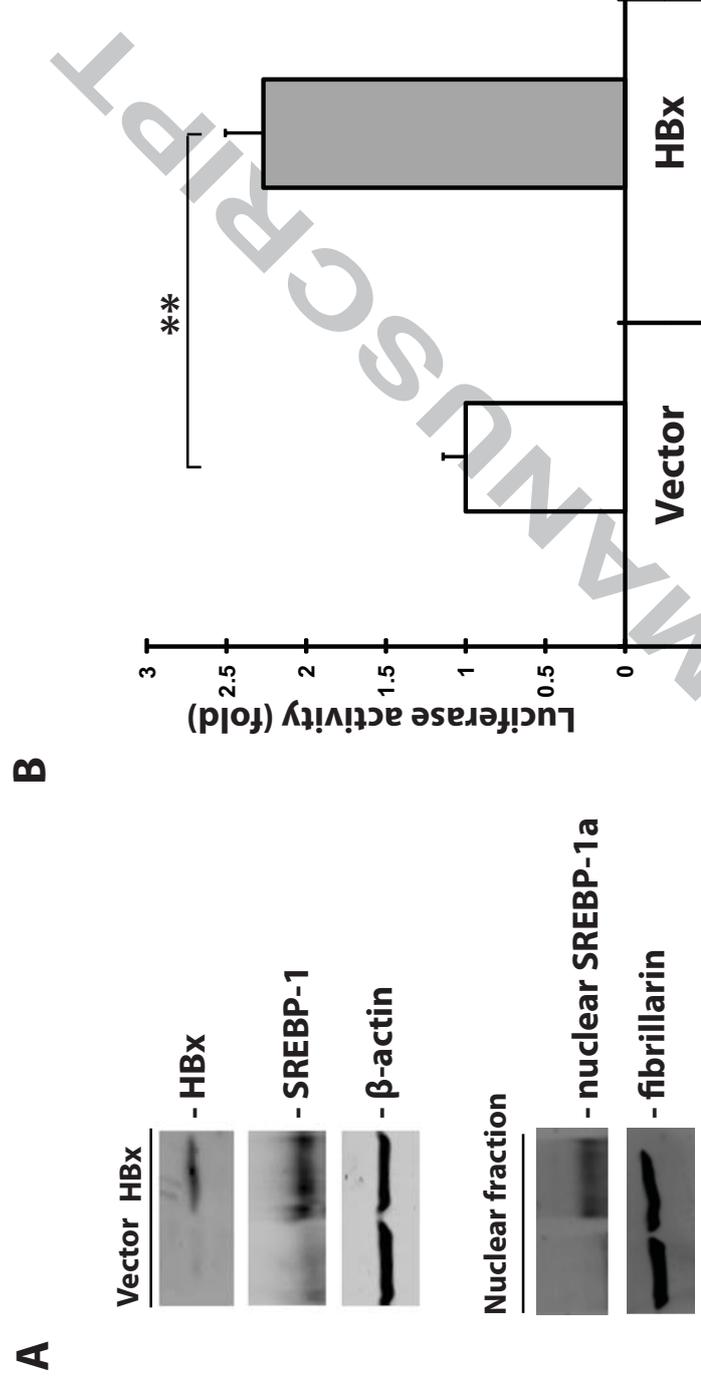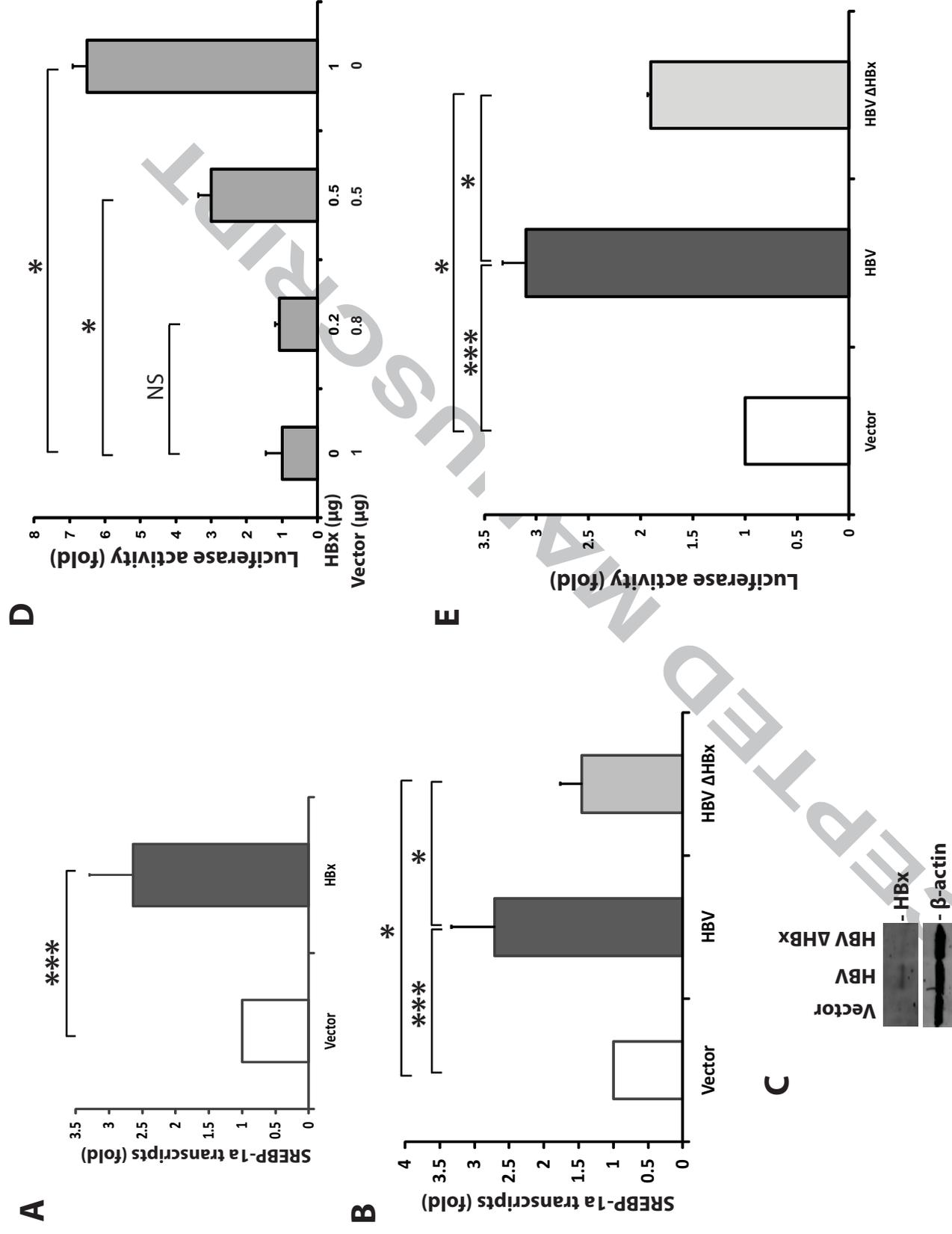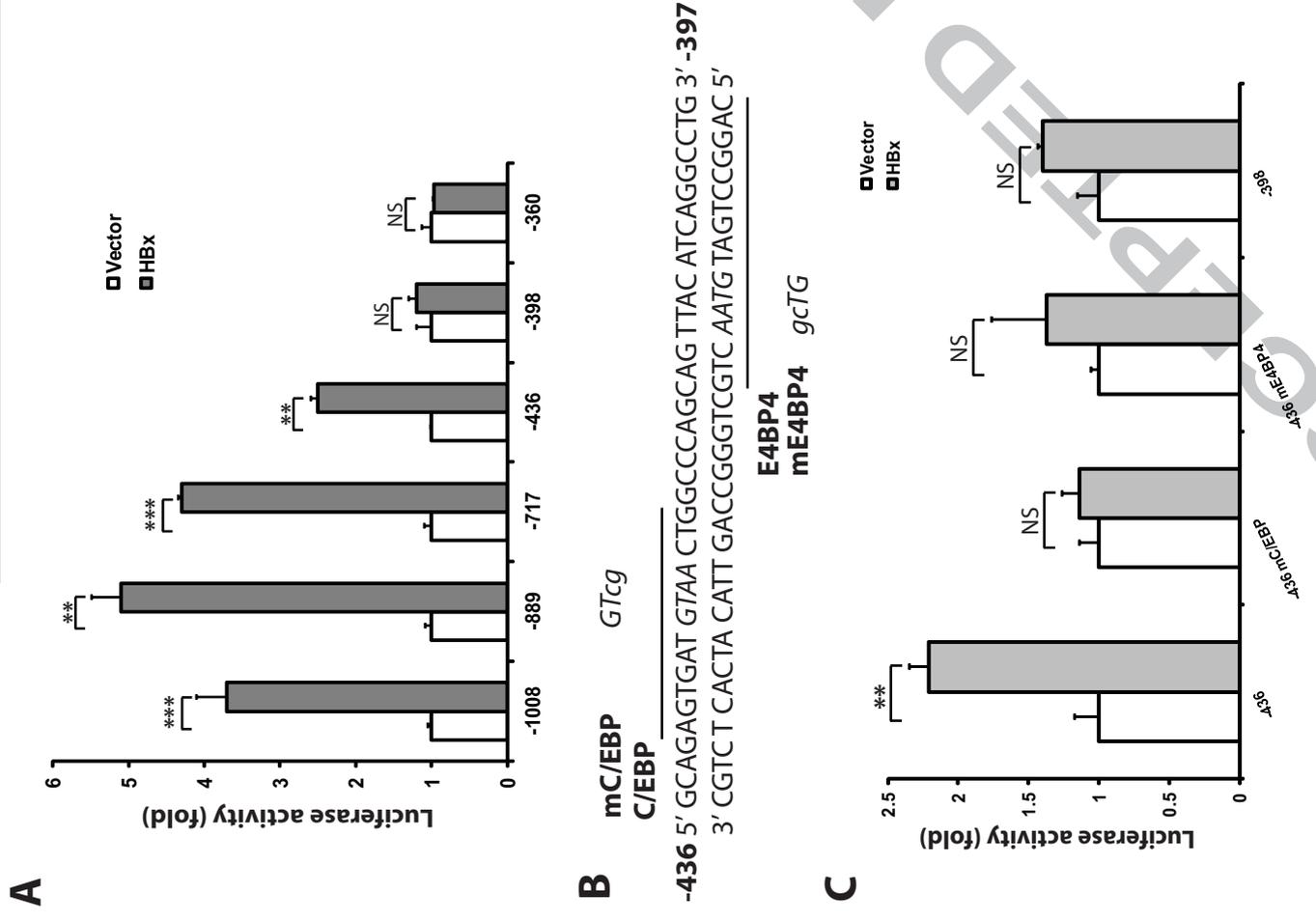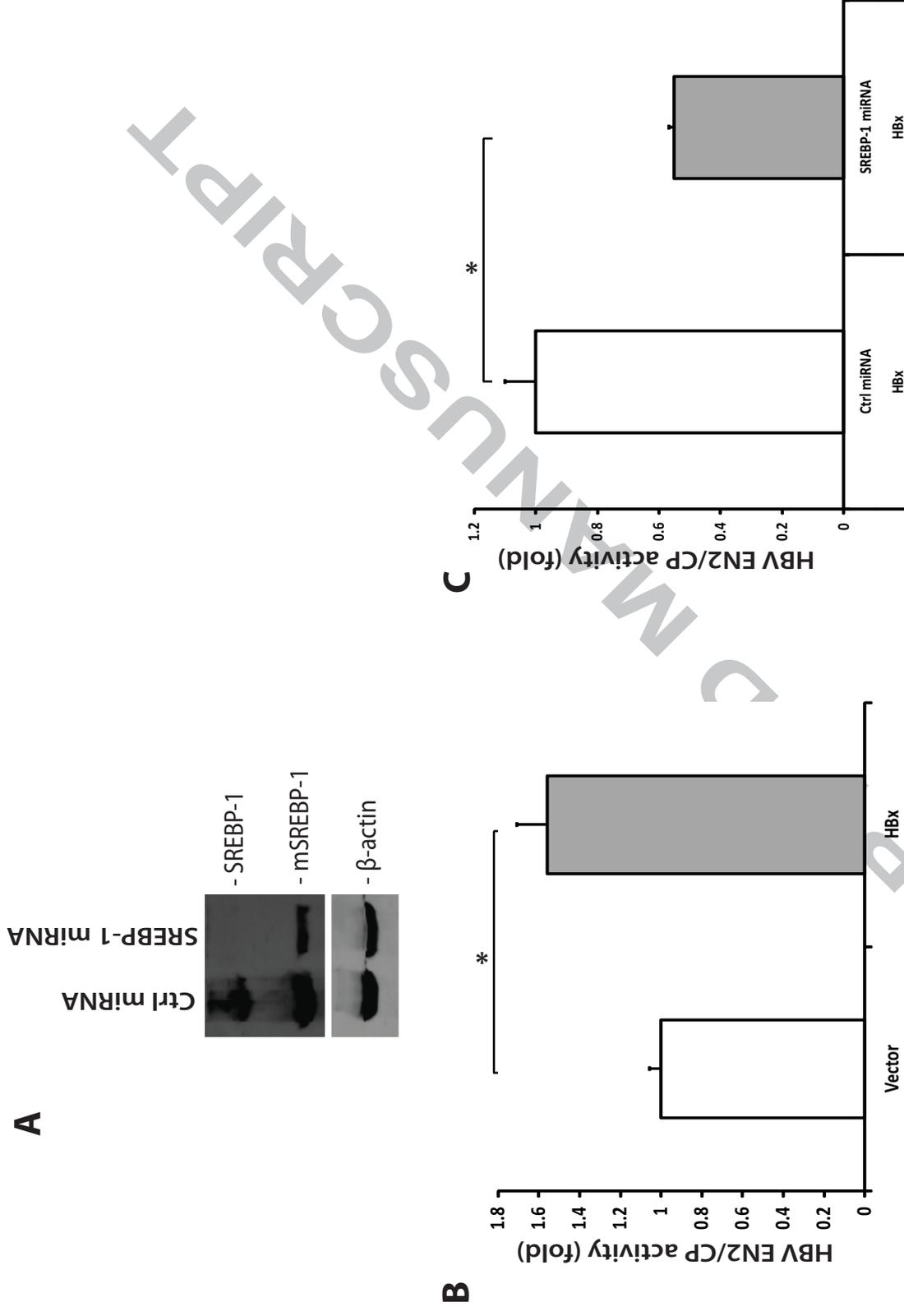
1. HBx increases mature SREBP-1a protein level

2. HBx activates SREBP-1a transcription

3. C/EBP and E4BP4 are involved in SREBP-1a promoter regulation by HBx

4. SREBP-1 is involved in HBV enhancer II/core promoter activation by HBx