Hepatitis B doubly spliced protein, generated by a 2.2 kb doubly spliced hepatitis B virus RNA, is a pleiotropic activator protein mediating its effects via activator protein-1- and CCAAT/enhancer-binding protein-binding sites

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The 2.2 kb doubly spliced defective hepatitis B virus (HBV) genome is frequently detected in the serum of patients with chronic hepatitis B. However, the biological significance of this type of defective genome is not well understood. In this study, expression of the hepatitis B doubly spliced protein (HBDSP) was confirmed from the 2.2 kb doubly spliced defective HBV genome, which was isolated and transfected into Huh-7 hepatoma cells. To explore the potential pathogenicity of HBDSP, hepatocellular proteins interacting with HBDSP were screened by a yeast two-hybrid assay. Unexpectedly, HBDSP could transactivate the GAL4-responsive element, and deletion mapping revealed that the fragment located between residues Leu-48 and Gin-75 of HBDSP was crucial for transactivation activity. In Huh-7 hepatoma cells, HBDSP localized predominantly to the cytoplasm and showed transactivating effects on the cytomegalovirus immediate-early promoter, simian virus 40 enhancer/promoter and HBV regulatory elements including the S1 promoter, S2 promoter, Enhancer I and core upstream regulatory sequences. Further studies revealed that the transactivating activities were mediated by activator protein-1- and CCAAT/enhancer-binding protein-binding sites. These findings suggest that HBDSP is a pleiotropic activator protein that can potentially serve as an HBV virulence factor.

INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus with a narrow host range that replicates via reverse transcription of a pre-genomic RNA intermediate. HBV infections can result in a broad spectrum of clinical manifestations (Bradley, 1999). Increasing evidence has revealed that heterogeneity of the HBV genome could alter basic aspects of the viral life cycle and influence the clinical course in patients (Hunt et al., 2000). Spliced variants of HBV are a group of defective DNA genomes generated from the 3.5 kb pre-genomic RNA of HBV by splicing and reverse transcription (Günther et al., 1995). Analysis of defective HBV genomes isolated from the sera of chronic hepatitis B patients revealed that the 2.2 kb spliced defective HBV genome is the most common (Günther et al., 1997) and is identified as singly (between nt 2447 and 489) or doubly (between nt 2447 and 2935 and between nt 3018 and 489) spliced isoforms depending on the splicing pattern.

The presence of the 2.2 kb singly spliced defective HBV genome has been shown to correlate with chronicity or persistence of HBV infection, liver fibrosis and lamivudine resistance (Rosmorduc et al., 1995; Soussan et al., 2003; Preiss et al., 2008). It was shown that the pathogenicity of this type of defective HBV is partially attributable to the encoded HBV spliced protein (HBSP) with multiple functions including inducing hepatocellular apoptosis (Soussan et al., 2000) and activating T-cell responses (Mancini-Bourgine et al., 2007).

Although the 2.2 kb doubly spliced HBV genome is also frequently detected in the serum of patients with chronic
hepatitis B, the biological significance of this type of defective genome remains unclear. In this study, the HBV doubly spliced protein (HBDSP) encoded by the 2.2 kb doubly spliced RNA was identified in transfected Huh-7 hepatoma cells, and its function was investigated further. We demonstrated that HBDSP is a pleiotropic activator protein mediating its effect via either activator protein-1 (AP-1) or CCAAT/enhancer-binding protein (C/EBP)-binding sites.

RESULTS

Isolation of the 2.2 kb doubly spliced defective HBV genome and expression of HBDSP in transfected Huh-7 hepatoma cells

PCR products ranging from 3.2 (full-length genome; Fig 1a) to 1 kb in length were recovered and cloned into the SacI sites of vector pUC19. From these clones, the 2.2 kb doubly spliced defective HBV genome was isolated and designated FD18-3 (genotype C, GenBank accession no. FJ151414.1). As shown in Fig. 1(b), the 5’ splicing sites of FD18-3 were located at nt 2447 and 3018, whilst the 3’ splicing sites were at nt 2935 and 489, resulting in the deletion of two separate regions from nt 2447 to 2935 (489 bp deletion) and nt 3018 to 489 (687 bp deletion), which encode the pre-S2, partial pre-S1 and S protein. The cis-acting sequences essential for transcription/encapsulation of the viral genome and functional regions including the pre-C/C and X genes were well conserved.

Notably, we found an ORF encoding a novel protein of HBDSP within the 2.2 kb doubly spliced HBV RNA, similar to that of HBSP derived from 2.2 kb singly spliced HBV RNA (Soussan et al., 2003). HBDSP was predicted to begin at the polymerase start codon and consisted of 139 aa. The region including Met1–Asn47 was homologous to the N-terminal region of the HBV polymerase protein, and that at residues Leu48–Gln75 was homologous to pre-S1; however, the C-terminal region (Glu76–Tyr139) resulting from a frameshift event was not homologous to any known HBV ORF (Fig. 1c). Importantly, we detected the ~15 kDa HBDSP protein by Western blot analysis of lysates from Huh-7 hepatoma cells transfected with LguI-released FD18-3 (Fig. 1d).

HBDSP transactivates the GAL4-responsive element, and the HBDSP48–75 domain is crucial for transactivation

HBDSP was used initially as a bait to screen for interacting hepatocellular proteins using a yeast two-hybrid system. To avoid false-positive results, it was necessary to evaluate the activation effect of HBDSP on the GAL4-responsive element before investing in a library search. Therefore, pGBK7-T-HBDSP encoding a GAL4 DNA-binding domain (BD)–HBDSP fusion protein was transformed into yeast AH109, and intracellular β-galactosidase activities were measured qualitatively. Unexpectedly, after the addition of substrate, a blue colour developed in AH109 cells transformed with pGBK7-T-HBDSP and pCL1 (positive control) within 2 and 1 h, respectively, whilst those with pGBK7-T-lam (negative control) and pGBK7 (blank control) remained colourless after 8 h (Fig. 2b, lanes 1–4). These results suggested that HBDSP can specifically transactivate the GAL4-responsive element.

To identify the HBDSP transactivating domain, full-length and various truncations of the gene encoding HBDSP (Fig. 2a) were cloned separately into pGBK7-T. Expression of the GAL4 fusion proteins in AH109 transformants was confirmed by Western blot analysis (Fig. 2c). Qualitative evaluation using a filter assay for intracellular β-galactosidase activity revealed that the pGBK7-T-HBDSP1–75, pGBK7-T-HBDSP48–75, pGBK7-T-HBDSP48–139 and pGBK7-T-HBDSP transformants turned blue within 2 h, whilst the pGBK7-T-HBDSP1–47, pGBK7-T-HBDSP76–139 pGBK7-T-
HBDSP-fusion transformants remained colourless after 8 h (Fig. 2b, lanes 5–10). The four positive transformants (pGBK7-HBDSP1–75, pGBK7-HBDSP 48–75, pGBK7-HBDSP48–139 and pGBK7-HBDSP) were selected for further quantitative measurement by liquid culture assay. The results demonstrated that the \( \beta \)-galactosidase activity level of each transformant was significantly higher than that of the empty vector control (\( P, 0.01 \), paired-samples \( t \)-test; Fig. 2d). Of note, HBDSP48–75 showed the maximal activity level, suggesting that aa 48–75, also present in HBDSP1–75, HBDSP48–139 and full-length HBDSP, are crucial for transactivation.

HBDSP can modestly transactivate the cytomegalovirus (CMV) immediate-early promoter and the simian virus 40 (SV40) promoter/ enhancer

To study the transactivation effects of HBDSP further, pcDNA3.1/HisC-HBDSP was constructed and two reporter plasmids, pCMV\( \beta \) and pSV-\( \beta \)-galactosidase, were introduced. Expression of HBDSP fusion proteins from Huh-7 cells transfected with pcDNA3.1/HisC-HBDSP peaked at 48 h post-transfection (Fig. 3a). Next, Huh-7 cells were transiently co-transfected with increasing amounts of effector plasmids of either pcDNA3.1/HisC-HBDSP or empty vector pcDNA3.1/HisC and with reporter plasmid pCMV\( \beta \) or pSV-\( \beta \)-galactosidase. The results showed that pcDNA3.1/HisC-HBDSP could induce the \( \beta \)-galactosidase activity of pCMV\( \beta \) or pSV-\( \beta \)-galactosidase in a dose-dependent manner, attaining a maximum effect of approximately twofold compared with empty vector at an effector : reporter plasmid ratio of 30 : 1 (\( P<0.01 \), paired-samples \( t \)-test; Fig. 3b, c), suggesting that HBDSP can modestly transactivate the CMV immediate-early promoter (pCMV\( \beta \)) and the SV40 promoter and enhancer (pSV-\( \beta \)-galactosidase).

Effects of HBDSP on HBV regulatory elements

To determine whether HBDSP has transactivation activity on viral genes, luciferase reporter plasmids driven by a
variety of HBV regulatory elements (Fig. 4a) were constructed and co-transfected with pcDNA3.1/HisC-HBDSP into Huh-7 cells. The intracellular luciferase activity was observed between cells transfected with pcDNA3.1/HisC-HBDSP and the empty pcDNA3.1/HisC control (P>0.05, paired-samples t-test) (Fig. 4b). Based on the above data, we concluded that HBDSP can transactivate CURS, Enhancer I, SP1 and SP2, but not BCP or XP.

**Transactivation of regulatory elements by HBDSP is mediated by C/EBP- and AP-1-binding sites**

Luciferase reporter plasmids driven by a variety of transcription recognition sequences were constructed and co-transfected separately with pcDNA3.1/HisC-HBDSP into Huh-7 cells. The results demonstrated that, with the C/EBP or AP-1 constructs, the intracellular luciferase activity of Huh-7 cells co-transfected with pcDNA3.1/HisC-HBDSP were 2–2.5 times greater than that of cells mock co-transfected with pcDNA3.1/HisC (P<0.01, paired-samples t-test) (Fig. 5). However, with nuclear factor-κB (NF-κB) transcription factor, Sp1 transcription factor, CCAAT-binding factor (CBF), hepatic nuclear factor 1 (HNF1), HNF3 and hepatocyte leukaemia factor (HLF) constructs, no differences were observed between cells transfected with pcDNA3.1/HisC-HBDSP and the empty pcDNA3.1/HisC control (P>0.05, paired-samples t-test). Taken together, these data indicated that transactivation of regulatory elements by HBDSP depends on C/EBP- and AP-1-binding sites.

**HBDSP localizes predominantly to the cytoplasm, endoplasmic reticulum and Golgi apparatus**

We first determined the subcellular distribution of HBDSP by Western blotting using an antibody to DsRed-Monomer to probe subcellular fractions of Huh-7 hepatoma cells transfected with pDsRed-HBDSP. The results showed that HBDSP was located predominantly in the cytosol, membrane organelles and cytoskeleton, and minimally in the nucleus (Fig. 6a). To gain further insight into the subcellular localization of HBDSP, confocal microscopy was used. When pDsRed-HBDSP was co-transfected with organelle-targeting GFP constructs, we observed that HBDSP localized mainly to the cytoplasm, endoplasmic reticulum and Golgi apparatus, but not to the mitochondria. Similar to the results of the Western blot analysis, HBDSP was observed at a relatively low level in the nucleus (Fig. 6b).

**DISCUSSION**

In the present study, the 2.2 kb doubly spliced defective HBV genome was isolated, and the expression of HBDSP was verified in transfected Huh-7 hepatoma cells. This protein could also be detected in other transfected hepatocyte-derived cell lines including Hep3B cells and transfects with pcDNA3.1/HisC (P<0.01, paired-samples t-test). By contrast, as observed for BCP1744–1851 and XP1239–1376, no differences in luciferase activity were observed between cells transfected with pcDNA3.1/HisC-HBDSP and the empty pcDNA3.1/HisC control (P>0.05, paired-samples t-test) (Fig. 4b). Based on the above data, we concluded that HBDSP can transactivate CURS, Enhancer I, SP1 and SP2, but not BCP or XP.
SMMC-7721 cells, and even hepatoblastoma HepG2 cells (data not shown). HBDSP, consisting of 139 aa, previously had unknown biological functions. During our initial attempt to determine the hepatocellular proteins that interact with HBDSP using a yeast two-hybrid screening system, we observed that HBDSP could transactivate the GAL4-responsive element. We then focused on understanding this function of HBDSP and found that the transactivating domain of the protein was located between residues Leu48 and Gln75.

HBV generates several transcriptional regulatory proteins including HBx (Twu & Schloemer, 1987; Rossner, 1992), the large surface antigen (Hildt et al., 1996), and the truncated middle surface protein (Hildt et al., 2002). These proteins display indirect transactivational activities towards many cellular and viral regulatory elements. In this study, we demonstrated that HBDSP could modestly transactivate the CMV immediate-early promoter, the SV40 promoter/enhancer and HBV regulatory elements including the SP1 and SP2, Enhancer I and CURS. To our knowledge, this is the first report describing the transactivating effects of an HBV spliced protein. More importantly, we found that the 2.2 kb doubly spliced defective HBV genome could be detected by PCR amplification in 52.5% (42/80) of chronic hepatitis B patients (data not shown). Nucleotide sequence alignment showed that the 5' splice sites at nt 2447 and 3018 and the 3' splice sites at nt 2935 and 489 are highly conserved among the eight known genotypes (A–H) of HBV (data not shown). Therefore, we concluded that
HBDSP is probably a conserved activator protein that potentially plays a role in HBV pathogenesis in the majority of chronic hepatitis B patients.

Interestingly, HBDSP showed a bias towards transactivation of SP1 and SP2, Enhancer I and CURS, but showed no effects on BCP and XP of HBV. It has been shown that there are several binding sites within the promoter/enhancer sequences for universal and hepatocyte-specific transcription factors, but the numbers and type of transcription factor-binding sites vary with different HBV regulatory elements (Moolla et al., 2002). To gain further insight into the possible mechanism of HBDSP transactivation of regulatory elements, several minimal promoter constructs harbouring binding sites for universal (AP-1, NF-κB, SP1 and CBF) and hepatocyte-specific (C/EBP, HNF1, HNF3 and HLF) transcription factors were each co-transfected with HBDSP. The results revealed that only AP-1 or C/EBP response element were required for promoter/enhancer activation by HBDSP. Therefore, it is reasonable that HBDSP can transactivate CURS containing two C/EBP-binding sites (Lopez-Cabrera et al., 1991) and Enhancer I containing one C/EBP-binding site (Dikstein et al., 1990) plus one AP-1-binding site (Lee et al., 1999), but not BCP and XP (Dikstein et al., 1990; Lopez-Cabrera et al., 1991; Lee et al., 1999) without binding sites for AP-1 and C/EBP. However, it was noteworthy that HBDSP could also transactivate SP1 and SP2 (Moolla et al., 2002), which do not contain binding sites for AP-1 and C/EBP. Therefore, the exact mechanism underlying the transactivation activities of HBDSP will require further study.

AP-1 family members (Jun/Jun or Fos/Jun) are thought to be involved in a diverse array of transcriptional regulatory processes governing cellular proliferation and differentiation and are associated with the development of a number of diseases (Karin et al., 1997). C/EBP-β induces gene transcription in response to gamma interferon (IFN-γ) through the IFN-γ-activated transcriptional element (Hu et al., 2001). It was reported previously that HBx may activate signal transduction cascades, resulting in transcriptional activation of cellular genes through cis-acting elements including C/EBP and AP-1 (Nijhara et al., 2001). We demonstrated here that the transactivation capacity of HBDSP is mediated through C/EBP- and AP-1-binding sites. However, because HBDSP localized primarily to the cytoplasm and did not seem to bind directly to AP-1- and C/EBP-recognition sites in an electrophoretic mobility shift assay (data not shown), we postulated that the transactivation capacity of HBDSP may depend on other signalling molecules in the cytoplasm or interaction with transcriptional factors in the nucleus; however, the exact mechanisms merit further study.

In summary, the HBDSP protein generated from the 2.2 kb doubly spliced viral RNA is an activator protein with pleiotropic but modest effects. With further studies to confirm the potential role of HBDSP in HBV pathogenesis, therapeutic interventions may be designed to inhibit the expression of this protein and thereby reduce virulence.

**METHODS**

**Amplification and cloning of HBV DNA.** Serum (100 μl) from a patient with chronic hepatitis B was incubated at 65 °C for 3 h with 300 μl TES buffer [10 mM Tris/HCl (pH 8.0), 5 mM EDTA, 0.5% SDS, 50 μg proteinase K]. DNA was extracted with phenol:chloroform (1:1) and subsequently with chloroform before precipitation with ethanol. HBV DNA was amplified by ‘hot-start’ PCR using high-fidelity Taq DNA polymerase (Roche) as described previously (Günther et al., 1997). The PCR products were recovered and cloned into the SacI site of vector pUC19. The cloned HBV genomes were sequenced, and the 2.2 kb doubly spliced defective HBV genome isolated in this study was designated strain FD18-3.
Antibody production and detection of HBDSP generated from the 2.2 kb doubly spliced hepatitis B virus RNA in Huh-7 hepatoma cells. Anti-HBDSP polyclonal antibodies were affinity purified after immunizing rabbits with a peptide corresponding to the C-terminal aa 76–87 of HBDSP with the sequence EHQLPARDHAKP (not homologous to any known HBV ORF) conjugated to keyhole limpet haemocyanin. Huh-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10 % FCS (Invitrogen). Recombinant plasmid DNA used for transfection was extracted and purified with a plasmid Maxi-prep kit (Qiagen), and the 2.2 kb doubly spliced defective HBV genome (FD18-3) was released from the recombinant plasmids by digestion with Lglu (MBI Fermentas) at 1 U (μg DNA)−1 for 16 h, followed by extraction and purification.

FD18-3 (50 μg) was transfected into Huh-7 cells in 60 mm plates using the calcium phosphate precipitation method as reported previously (Lin et al., 2001). An equal amount of Lglu-digested pUC19 DNA was used as a mock transfection control. The reporter plasmid pSEAP2-Control (3 μg; Clontech) expressing secreted alkaline phosphatase (SEAP) was used to co-transfect as an internal control for the normalization of transfection efficiency. The medium was changed at 12 h and the cells harvested at 96 h post-transfection. Cells were washed twice with pre-chilled PBS and lysed using RIPA buffer [50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 0.1 % SDS, 0.5 % deoxycholate, 1 % NP-40, 2 mM EDTA, 50 mM Na3VO4] containing Protease Inhibitor Cocktail (Roche). The protein concentration of cell extracts was determined by the Bradford method (Bio-Rad). SEAP-normalized protein (20 μg) was subjected to 15 % PAGE, transferred to PVDF membranes (Millipore) and incubated with anti-HBDSP polyclonal antibody (1:50 dilution). The primary antibodies were detected by alkaline phosphatase-conjugated secondary antibodies, and the immunoreactive protein bands were visualized using CDP STAR reagents (Roche).

Construction of HBDSP expression vectors. The 2.2 kb doubly spliced defective HBV genome (strain FD18-3) was used as a cloning template. The predicted translation beginning at the polyserase start codon would result in an HBDSP product containing 139 aa. For the construction of pCDNA3.1/HisC-HBDSP, the HBDSP ORF was amplified by PCR and cloned into the EcoRI and NotI sites of pCDNA3.1/HisC (Invitrogen). For the construction of yeast expression vectors, full-length and various truncations of the HBV gene were generated by PCR amplification (with the exception of the HBDSP fusion segment) and cloned into the EcoRI and PstI sites of pGBKT7 (bait plasmid with a GAL4 DNA-BD; Clontech). The primers used were P1–P8 as described in Supplementary Table S1 (available in JGV Online) and Fig. 2(a). The constructs generated were pGBKT7-HBDSP (encoding full-length HBDS), pGBK7-HBDSP1–147 (Met1–Asn47), pGBK7-HBDSP76–139 (Met1–Gln75), pGBK7-HBDSP148–190 (Leu48–Gln75), pGBK7-HBDSP191–195 (Leu48–Tyr139), pGBK7-HBDSP206–212 (Glu76–Tyr139) and pGBK7-HBDSP-fusion (Met1–Asn47 plus Glu76–Tyr139). To construct the red fluorescent protein-fused HBDSP, the HBDSP ORF was cloned into the Xhol and SalI sites of pDsRed-Monomer-Hyg-N1 (Clontech) to generate pDsRed-HBDSP.

Construction of luciferase reporter plasmids. The template used for generation of luciferase reporter constructs was 1.2 × HBV DNA (strain 56, genotype B, GenBank accession no. AF100309) (Lin et al., 2001; Huang et al., 2009). HBV regulatory elements (Moolla et al., 2002) including BCP1744–1851, CURS, XP1239–1376, XP/EnhI 1071–1376, SP12720–2809 and SP22984–3215 were amplified by PCR using the primers described in Supplementary Table S1 and Fig. 4(a). All of the PCR products were cloned into pGL3-Basic (Promega) using the KpnI and Xhol restriction sites. The constructs generated were designated pGL3-CP, pGL3-BCP1744–1851, pGL3-BCP1912–2002, pGL3-XP, pGL3-EnhI1071–1376, pGL3-SP12720–2809 and pGL3-SP22984–3215.

The cis-luciferase reporter plasmids were generated using pLuc-MCS (Stratagene). The inserts included binding sites for seven copies of AP-1, three copies of Sp1 transcription factor, four copies of CBF, three copies of C/EBP, five copies of NF-kB transcription factor, two copies of HNF1 (Li & Klaassen, 2004), four copies of HNF3 (Raney et al., 1995) and four copies of HLF (Ishida et al., 2000). The inserts were obtained by annealing two oligonucleotides to form a double-stranded segment of DNA with a 5’ single-stranded overhang at both ends and cloned into the HindIII and Xhol sites of pLuc-MCS. The oligonucleotide sequences used are listed in Supplementary Table S2.
Yeast transformation and detection of GAL4 fusion proteins. AH109 yeast strains were cultured in complete YPD medium (Clontech) at 30 °C. Each of the HBDSP constructs, empty pGBK7T, negative-control pGBK7T-Jam or positive-control pGCL (Clontech) were transformed separately into AH109 cells using polyethylene glycol/lithium acetate following the instructions of the manufacturer (Clontech). Intracellular total proteins from AH109 transformants were extracted using the SDS/urea method as described by the manufacturer (Clontech) and then subjected to 15% SDS-PAGE. The blots were transferred to PVDF membranes and probed with anti-GAL4 DNA-BD monoclonal antibody (1:4000 dilution; Clontech).

β-Galactosidase assays of AH109 transformants. The intracellular β-galactosidase activities of yeast transformants were measured qualitatively and quantitatively. Qualitative evaluations of the intracellular β-galactosidase activity of yeast transformants were carried out using a filter assay as described previously (Lei et al., 1999). Briefly, yeast cells streaked on filters were then placed on another piece of filter paper pre-soaked with a Z buffer/X-Gal solution (Z buffer with 0.8 mM X-gal) and 0.27 % yeast-streaked filters were then placed on another piece of filter paper thaw cycles (between liquid nitrogen and room temperature). These yeast-streaked filters were then placed on another piece of filter paper pre-soaked with a Z buffer/X-Gal solution (Z buffer with 0.8 mM X-Gal and 0.27 % (v/v) β-mercaptoethanol; Z buffer: 0.06 M Na2HPO4·7H2O, 0.04 M NaH2PO4·H2O, 0.01 M KCl, 0.001 M MgSO4·7H2O) and checked for blue color development every 30 min for 8 h at 30 °C. Quantitative analysis was carried out using a liquid culture assay following the manufacturer’s instructions (Clontech). Briefly, each transformant was inoculated into liquid medium; the cells were harvested when the OD600 value reached 0.5–0.8 and disrupted by freeze/thaw cycles (between liquid nitrogen and room temperature). These yeast-streaked filters were then placed on another piece of filter paper pre-soaked with a Z buffer/X-Gal solution (Z buffer with 0.8 mM X-Gal and 0.27 % (v/v) β-mercaptoethanol; Z buffer: 0.06 M Na2HPO4·7H2O, 0.04 M NaH2PO4·H2O, 0.01 M KCl, 0.001 M MgSO4·7H2O) and checked for blue color development every 30 min for 8 h at 30 °C. Quantitative analysis was carried out using a liquid culture assay following the manufacturer’s instructions (Clontech). Briefly, each transformant was inoculated into liquid medium; the cells were harvested when the OD600 value reached 0.5–0.8 and disrupted by freeze/thaw cycles (as above). Z buffer with 0.27 % (v/v) β-mercaptoethanol and 2 mM o-nitrophenyl β-D-galactopyranoside was added to the yeast cell lysates, followed by incubation of the reaction tubes in a 30 °C water bath. The reaction was stopped by adding 1 M Na2CO3 when a yellow color was detected in the tubes, and the elapsed incubation time was recorded in minutes. At the final step, the OD420 value was detected using a spectrophotometer (Bio-Rad), and intracellular β-galactosidase units were calculated as 1000 × OD420(1 × 0.5 × OD600) where t represents the elapsed incubation time (min). Experiments were performed five times, and data are shown as means ± s.d.

Reporter gene assays of co-transfected Huh-7 hepatoma cells. Two micrograms of pcDNA3.1/HisC-HBDSP or the pcDNA3.1/HisC empty vector were transfected separately into Huh-7 hepatoma cells using FuGENE6 Transfection Reagent (Roche). The medium was changed 6 h after transfection, and the cells were harvested at 24, 48, 72 and 96 h post-transfection. Cells were washed twice with pre-chilled PBS and lysed using RIPA buffer containing Proteinase Inhibitor Cocktail. The protein concentrations of the cell extracts were determined by the Bradford method (Bio-Rad). The proteins (20 μg) were subjected to 15% SDS-PAGE and probed with anti-Xpress (detects the Xpress tag expressed by pcDNA3.1, diluted 1:2000 dilution; Invitrogen) or anti-β-actin antibody (1:4000 dilution; Sigma).

For β-galactosidase assays, pcDNA3.1/HisC-HBDSP was co-transfected with 0.05 μg pCMVβ (β-galactosidase driven by the CMV immediate-early promoter; Clontech) or 0.1 μg pSPV-β-galactosidase (β-galactosidase driven by the SV40 promoter/enhancer; Promega) at ratios ranging from 10:1 to 50:1. The DNA used for each transfection was adjusted using vector pUC19 DNA to normalize the total amount of DNA. Cells were harvested and lysed at 48 h post-transfection, and 20 μg protein was used for the detection of intracellular β-galactosidase activity (β-Galactosidase Enzyme Assay System; Promega) following the manufacturer’s instructions. Each transfection was performed in duplicate and repeated five times.

For luciferase assays, 1 μg pcDNA3.1/HisC-HBDSP was separately co-transfected with equal amounts of pGL3-CP1601–1851, pGL3-XP/Enh1071–1376, pGL3-XP1239–1376 or pGL3-SP2964–3215, or with 3 μg pGL3-BCP1744–1851 or pGL3-SP12720–2809. These latter two promoters were previously shown to display relatively weak activities (Nakatake et al., 1993). Cells were harvested and lysed at 48 h post-transfection, and 20 μg protein was used for the detection of intracellular luciferase activity (Bright-Glo Luciferase Assay System; Promega). The light intensity was measured by a luminometer (Orion II Microplate Luminometer; Berthold Detection Systems). The RLU were obtained by comparison with the transfected HBV regulatory element only, which was set to a value of 1 in each experiment. Each transfection was performed in duplicate and repeated five times.

Isolation of subcellular fractions and Western blot analysis of HBDSP. Huh-7 hepatoma cells were transfected with 3 μg pDsRed-HBDSP, and subcellular fractions of the cytosol, membrane organelles, nucleus and cytoskeleton were isolated at 48 h post-transfection using a ProteoExtract Subcellular Proteome Extraction kit (EMD Biosciences) following the manufacturer’s instructions. Protein samples were separated by 10% SDS-PAGE, transferred to PVDF membrane and detected with an anti-DsRed polyclonal antibody (1:2000 dilution; Clontech).

Confocal fluorescence microscopy. Huh-7 hepatoma cells were seeded onto coverslips in 60 mm plates 24 h prior to transfection and incubated overnight to ensure cultures of ~50% confluency. A set of vectors expressing GFP targeting to the cytoplasm (pCMV/Myc/Cyto/GFP; Invitrogen), mitochondria (pCMV/Myc/mito/GFP; Invitrogen), endoplasmic reticulum (pCMV/Myc/ER/GFP; Invitrogen) or Golgi apparatus (pAcGFP-Golgi; Clontech) were co-transfected separately with pDsRed-HBDSP. At 48 h post-transfection, cells were fixed with 2% paraformaldehyde for 15 min at 37 °C, neutralized in 50 mM NH4Cl, permeabilized in 0.4% Triton X-100 and incubated with Hoechst 33342 at a final concentration of 3 μg ml−1 for 1 h at 37 °C. Stained cells were then visualized on a Zeiss LSM 510 NLO confocal microscope.

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