Hepatitis B Virus-induced hFGL2 Transcription Is Dependent on c-Ets-2 and MAPK Signal Pathway*

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Fibrinogen-like protein 2 (FGL2)/fibroleukin plays a pivotal role in the pathogenesis of experimental and human fulminant and chronic viral hepatitis. To define the transcription factor(s) and upstream signal transduction pathways involved in the transcription of human FGL2 (hFGL2) in response to hepatitis B (HB) virus, hepatitis B core (HBc), hepatitis B virus S protein (HBs), or hepatitis B virus X protein (HBx) protein, expression plasmids were cotransfected with an hFGL2 promoter luciferase reporter construct into Chinese hamster ovary and HepG2 cells, respectively. HBc and HBx proteins, but not HBs protein, enhanced hFGL2 transcription in both cell lines. A strong regulatory region from −712 to −568 (relative to the transcriptional starting site) was shown to be responsible for hFGL2 gene transcription in response to both HBc and HBx proteins. c-Ets-2 was shown to be translocated to the nucleus in association with hFGL2 expression in response to both HBc and HBx proteins. Short hairpin RNA (shRNA) interference of c-Ets-2 expression inhibited hFGL2 gene transcription by 64.8 and 60.0% in response to HBc and HBx, respectively. c-Ets-2 protein was highly expressed in peripheral blood mononuclear cells from patients with severe chronic hepatitis B (CHB) in contrast to patients with mild CHB. Increased phosphorylation of ERK and JNK was detected in peripheral blood mononuclear cells from patients with severe CHB. ERK inhibitor PD908509 or ERK shRNA abolished the nuclear c-Ets-2 DNA binding activity and hFGL2 induction in response to HBc, whereas JNK inhibitor SP600125 or JNK shRNA abolished the nuclear c-Ets-2 DNA binding activity and hFGL2 induction in response to HBx. In conclusion, HBc and HBx proteins enhance transcription of hFGL2 through c-Ets-2-dependent on MAPK signal pathways.

Hepatitis B virus (HBV) is a hepatotropic DNA virus with four open reading frames encoding four proteins, including hepatitis B core (HBc), surface (HBs), X protein (HBx), and DNA polymerase. HBV-encoding proteins play important roles in the pathogenesis of acute and chronic liver disease, including HBV-induced acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1–3). These encoding proteins only are important in viral replication but are also capable of regulating host gene expression (3–9). It has been reported that HBx represses the p53 promoter through an E-box (10), and HBx may play a role in hepatocellular carcinogenesis by interfering with telomerase activity during hepatocyte proliferation (9). HBx has also been reported to induce matrix metalloproteinase-9 gene expression through activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathways (5). HBc protein is a potent inducer of IL-18 in peripheral blood mononuclear cells (PBMC) of healthy controls and patients with hepatitis B infection (4) and represses the transcription of the human p53 gene in human liver cells (8). These findings indicate that HBV proteins regulate host genes expression, which in turn may influence the course and severity of hepatic inflammation.

Fibrinogen-like protein 2 (FGL2)/fibroleukin, also known as fgl2 prothrombinase, has been cloned and identified to belong to the fibrinogen protein superfamily. Mouse Fgl2 (mFgl2) and human FGL2 (hFGL2) have been localized to chromosomes 5 and 7, respectively (11–13). FGL2 prothrombinase has a serine protease activity and is capable of directly cleaving prothrombin to thrombin leading to fibrin deposition (14). Previous work has demonstrated that mFgl2/hFGL2 selective expression in the liver plays a pivotal role in the pathogenesis of both experimental and human fulminant viral hepatitis and severe chronic hepatitis B (CHB) (15–20), and genetic interference of mFgl2 gene leads to significant increase of survival for mice with fulminant viral hepatitis (21). mFgl2 and hFGL2 gene expression is confined to macrophages, lymphocytes, and endothelial cells of
intrahepatic veins and hepatic sinusoids (17, 22). In humans, expression of hFGL2 transcripts and protein was detected in liver as well as PBMC in patients with severe CHB, whereas only basal low expression of hFGL2 was seen in healthy controls or patients with mild CHB (16, 23). Although we have reported a critical role for hFGL2 in the pathogenesis of severe CHB, the underlying mechanism for its increased expression is unknown. The expression of mFgl2 mRNA transcription and protein is induced by IFN-γ in macrophages and TNF-α in endothelial cells, respectively (24). Other factors have also been reported to contribute to mFgl2 transcription. It has been reported that the nucleocapsid protein of mouse hepatitis virus 3 (MHV-3) induces transcription of mFgl2 and invokes hepatic nuclear factor 4α in mFgl2 gene expression (20, 25). MHV-3 induces mFgl2 gene transcription through p38 mitogen-activated protein kinase (MAPK) activation (26). Previous data has shown that hFGL2 and mFgl2 have 70% homology (16). We therefore postulate that hFGL2 gene transcription is regulated by encoded proteins of HBV, the most frequent cause of liver failure in Asia.

In this study, we demonstrate that Hbc and HBx initiate the transcription of hFGL2 gene through the transcription factor c-Ets-2 dependent on the activation of ERK and c-Jun N-terminal kinase (JNK) signal pathways in response to either Hbc or HBx proteins. Furthermore, we demonstrated increased c-Ets-2 expression and activity of ERK and JNK in PBMC in association with severe CHB viral infection.

**MATERIALS AND METHODS**

**Creation of HBs, Hbc, and HBx Gene Eukaryotic Expression Plasmids**—HBV DNA with HBV genotype B was isolated from serum of an informed patient with HBV DNA positive and used as template for PCR amplification to obtain Hbc, HBs, and HBx entire gene. Three pairs of primers, C1/C2, S1/S2, and X1/X2 (C1, 5’-TTT GGG GCA TGG ACA TTG AC-3’, and C2, 5’-CCC ACC TTA TGA TGC CAA GG-3’; S1, 5’-AAC ATG GAC AAC ATC ACA TC-3’, and S2, 5’-CAA CTG CCA ATG ACA TAA CC-3’; and X1, 5’-AGC GAA TTC CAT GGC TGC TAG GCT G-3’, and X2, 5’-CGC TCT AGA TTG GAG TCT TGA ACA G-3’), were used accordingly to amplify Hbc, HBs, and HBx genes with the products of 583, 747, and 520 bp, respectively. Hbc, HBs, and HBx genes were cloned into PCR2.1 cloning vector (Invitrogen) and then subcloned into HindIII/XhoI sites to form six 5’ deletion hFGL2 promoter/luciferase reporter constructs. The promoter luciferase report plasmids were all sequenced to confirm the orientation and to verify the sequence.

**Transient Transfection**—Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) for CHO and HepG2 cells and Lipofectamine LTX (Invitrogen) for THP-1 cells according to the manufacturer’s instruction. 1 μg of expressing plasmid pcDNA-HBc or pcDNA-HBs or pcDNA-HBx, 1 μg of hfgl2p(−1334)LUC DNA, 0.5 μg of β-galactosidase DNA (as a marker for transfection efficiency) in 100 μl of Opti-MEM medium (Invitrogen) were mixed by vortexing with 4 μl of Lipofectamine 2000 in 100 μl Opti-MEM medium. After incubation of the mixture at room temperature for 20 min, 0.8 ml of Opti-MEM medium (Invitrogen) was added to bring up the volume to 1 ml. 1 ml of the mixture was distributed into 1 well with CHO cells or HepG2 cells. After cells were cultured for 10–12 h, another 1 ml of culture medium was added to the well. Transfection was performed at 37 °C with 5% CO2 for 36–48 h.

**Immunocytochemistry**—CHO cells transfected with expressing vector pcDNA-HBc or pcDNA-HBs were stained with the anti-Hbc or anti-HBs polyclonal antibody from rabbit (1:200 dilution; Santa Cruz Biotechnology) overnight followed by incubation with a secondary biotinylated anti-rabbit IgG (1:200 dilution). Solution of streptavidin-peroxidase was then added. As negative controls, CHO cells, either without transfection or transfected with pcDNA3.1(+) empty plasmid, were employed.

**Western Blotting Analysis**—CHO cells transfected with pcDNA-HBc were lysed in a lysis buffer containing 150 mm NaCl, 50 mm Tris, pH 7.4, 5 mm EDTA, 1% Nonidet P-40, and protease inhibitors for 30 min on ice, and whole cell lysate was obtained by subsequent centrifugation. Samples were heated at 100 °C for 5 min and cooled on ice. Fifty micrograms of protein from whole cell lysates was subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Blocking was performed in 5% (w/v) nonfat dried milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and then incubated with specific monoclonal antibody against HBx (1: 400 dilution, Usbio Technology).

**Real Time Fluorescence Quantitative RT-PCR and Western Blotting Analysis of hFGL2 Expression**—Total RNA (1 μl) from THP-1 cells transfected with pcDNA-HBc, pcDNA-HBs, or pcDNA-HBx was isolated and reverse-transcribed into cDNA using TRIzol reagent (Invitrogen) and reverse transcriptase (Fermentas) according to the manufacturer’s standard protocol. Real time fluorescence quantitative RT-PCR was done with EvaGreen PCR reagents (Biotium) according to the manufacturer’s standard protocol to detect hFGL2 and c-Ets-2 expression at the mRNA level. The upstream primer was 5’-ACT GTG ACA TGG AGA AGA TTG CTT TTG GAT G-3’ for hfgl2p(−1334)LUC; 5’-AGC GAC ACT GGT TTT GAT G-3’ for hfgl2p(−712)LUC; 5’-TGA AGG AAA ATG CAA CTG C-3’ for hfgl2p(−568)LUC; 5’-AAT ACA GGC TCC CCA ATG C-3’ for hfgl2p(−467)LUC; and 5’-GTG AAT CTT GTT GGC TGG GCT G-3’ for hfgl2p(−243)LUC. The common downstream primer used was 5’-TTC TGC GCA GAT CAT TTG C-3’.
and the proteins were detected with purified polyclonal antibody against hFGL2.

Luciferase Assay—After transfection for 44–48 h, luciferase activity and β-galactosidase activity were assayed by using the luciferase and β-galactosidase enzyme assay systems (Promega) in CHO cells and HepG2 cells. Luciferase activity was normalized with the β-galactosidase activity in cell lysate and calculated as an average of three independent experiments. PGL2-Basic vector was used as a negative control.

Site-directed Mutagenesis and Luciferase Assays—Constructs bearing mutant promoter variants of the hFGL2 gene were generated by PCR using the wild type hFGL2 promoter/luciferase report construct hfgl2p(-1334)LUC as template according to the manufacturer’s protocol in the QuickChange™ site-directed mutagenesis kit (Stratagene). Primers were designed according to manufacturer’s instructions and produced by Invitrogen as shown in Table 1. The boldface and underlined letters indicate the mutant sequences. All constructs generated were sequenced to confirm the orientation and to verify the sequence.

| Mutants name | Sequence change | Sense primer sequence (5‘-3‘) | Anti-sense primer sequence (5‘-3‘) |
|--------------|-----------------|-----------------------------|----------------------------------|
| Nks-2mut     | AATTAT to GCCCG | Sense, CAG CTA CTG TTC GTG ATG AAA GAC | AGA C TGG GAC CCA TTC AAA AGG ACT CAT GCT |
| LEP-1/Etsmut | AGG to CACG    | Sense, CAC TAC GCT ACG GAC AAC | AC CGG TTC CTT GAT TTC ACT TAC ACG |
| HSTmut       | AGG to GCG     | Sense, CTA GTG GAG AAA AAA | GGA GAA GAA AAGC CCG GAG GGA AGA AAG |
| SRYmut       | TAG to GCC      | Sense, CAA TAT GCT ACG GAC AAA | GAA GAA GAA AAGC CCG GAG GGA AGA AAG |
| Evi-1mut     | CTT to GCC     | Sense, CTA GTG GAG AAA AAA | GGA GAA GAA AAGC CCG GAG GGA AGA AAG |

Mutations were made to the promoter sequence of the hFGL2 gene by a site-directed mutagenesis protocol as described under “Materials and Methods.” Sense and antisense primers were designed to encode the desired mutations. The underlined and boldface letters indicate the mutant sequences. All constructs generated were sequenced to confirm the orientation and to verify the sequence.

Below is the table for the primer pairs used to construct hFGL2 promoter mutants:

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TCT TCG CAT TTC CCT CT-3'. The amount of target IP DNA was quantified on an ABI7900 HT sequence detection system (Applied Biosystem) using SYBR green methodology.

Confocal Microscopy—After blocking, the transfected and nontransfected THP-1 cells were first incubated with a polyclonal antibody from rabbit against the c-Ets-2 protein (Santa Cruz Biotechnology) at 4 °C temperature for 12 h followed by five washes in PBS. Cells were then incubated with a fluorescent isothiocyanate-conjugated goat IgG fraction against the rabbit IgG Fc fragment at room temperature for 1 h, followed by five washes with PBS. Then RNase was added to digest the RNA in the cells. The slides were stained by propidium iodide (50 μg/μl) for 30 min followed by five washes with PBS.

shRNA-based Experiments—We constructed an shRNA plasmid for c-Ets-2 using retroviral vector pMSCVneo. The sequence of target for human c-Ets-2 was AAA CAC TGC AAG AAG TGC CAA. The sequence scramble of control shRNA was AGG TGC AAC AAC AAT GCA CAA. Mouse U6 promoter was amplified from mouse genmic DNA. We then cloned U6 promoter following sequences of hairpin of target gene into pMSCVneo to form shRNA interfering plasmids named c-Ets-2 shRNA and control shRNA, respectively. The c-Ets-2 shRNA construction was sequence-verified. After transfection with c-Ets-2 shRNA, RT-PCR and real time quantitative RT-PCR analysis were performed to determine c-Ets-2 expression at the mRNA level using the forward primer 5'-CGG AAG CTT AGA ATA TGG ACC AGG TAG CC-3' and the reverse primer 5'-TAA GGA TCC GTT CCT TGC CAA GGT TAC AC-3'. For Western blotting, protein extracts from the same number of cells were loaded and were separated on a 10% SDS-polyacrylamide gel, and the proteins were detected with anti-c-Ets-2 (Santa Cruz Biotechnology). To detect the effect of c-Ets-2 shRNA on the hFGL2 transcription, 1 μg of hFGL2 luciferase report plasmid, 1 μg of HBV protein expression plasmid, 0.5 μg of β-galactosidase DNA, and 1 μg of shRNA were mixed and transfected into THP-1 cells based on the transfection manuals.

Studies in Patients with Viral Hepatitis B—Informed consent was obtained from human study participants, and the research protocol was reviewed and approved by the institutional review board of Tongji Hospital, Wuhan, China. Patients were recruited at Tongji Hospital, Hubei Province, China. Biochemical, histological, and clinical features were used to define

**FIGURE 1.** Expression of pcDNA-HBc, pcDNA-HBs, and pcDNA-HBx in CHO cells. A and B, CHO cells transfected with pcDNA-HBc or pcDNA3.1 (control) were stained with antibody for HBc by immunohistochemistry; C and D, CHO cells transfected with pcDNA-HBs or pcDNA3.1 (control) were stained with antibody for HBs by immunohistochemistry. E, Western blotting analysis of HBx expression. CHO cells transfected with pcDNA-HBx or pcDNA3.1 were measured for protein expression by using anti-HBx antibody. β-Actin was detected as internal control for equal loading of the protein samples.

**FIGURE 2.** HBc and HBx induce activation of hFGL2 prothrombinase. Effect of HBc and HBx protein on hFGL2 mRNA and protein expression in vitro was determined by quantitative real time RT-PCR in A and Western blotting in B. Values in A represent the mean and S.D. of three separate experiments done in triplicate. * represents p < 0.05 compared with untreated group. C, HBc and HBx induce transcriptional activity of hFGL2 promoter. After cotransfection for 44–48 h, CHO and HepG2 cells were harvested and lysed for measurement of luciferase activity. Value represents the mean ± S.D. of three separate experiments done in triplicate. * represents p < 0.01 compared with cells cotransfected with pcDNA3.1 empty vector.
patients with chronic viral hepatitis B (severe chronic viral hepatitis B versus mild chronic viral hepatitis B infection). Standard definitions for diagnostic criteria and laboratory investigations were used as described previously (17, 19). Percutaneous liver biopsies were obtained for the purposes of guiding treatment decisions (28). The methodology for hepatitis B viral markers, serology, and hepatitis B viral DNA loads and the collection of specimens were described previously (17). Twenty two patients with severe CHB, 14 patients with mild chronic viral hepatitis B, and 9 HBsAg carriers were evaluated (Table 2). PBMCs were freshly isolated using Ficoll-Hypaque gradient centrifugation from the above-mentioned patients and six health controls and were smeared on slides and kept at –80 °C for further study. PBMCs were evaluated for confocal microscopic study and Western blotting to detect the c-Ets-2 expression as described above.

**Flow Cytometry Analysis**—The expression level of c-Ets-2 in PBMC of patients with severe CHB and mild CHB was confirmed by flow cytometry. A total of 10⁶ cells were incubated with 0.5 μg of antibody against c-Ets-2 in staining buffer (1% bovine serum albumin in PBS) for 10 h at 4 °C. Cells were washed and incubated with a fluorescent isothiocyanate-conjugated goat IgG fraction against the rabbit IgG Fc fragment at room temperature for 1 h. The cells were washed in staining buffer, and fluorescence was measured using a FACScalibur flow cytometry (Pharmingen).

**Detection of Phosphorylated JNK, ERK, and p38 MAPK in PBMC from Patients by Western Blotting**—The level of active forms of JNK(P-JNK), ERK(P-ERK), and p38 MAPK (P-p38 MAPK) in the PBMC lysates (5 μg each) were examined separately by Western blotting with anti-phospho plus stress-activated protein kinase/JNK, P-ERK antibody, and P-p 38 MAPK antibody (Cell Signaling Technology), respectively. Respective blots were stripped and reprobed with anti-JNK, anti-ERK, and anti-p38 MAPK antibody.

**Detection of HBc- and HBx-mediated Activation of ERK and JNK**—THP-1 cells were transfected with pcDNA-HBc or pcDNA-HBx for 0, 12, 24, 36, and 48 h, and then cell lysates were extracted for Western blotting to detect the activation of ERK, JNK, and p38 MAPK and the expression of c-Ets-2 and hFGL2.

**Translocation of c-Ets-2 in THP-1 Cells**—The cytoplasm and nucleoplasm of THP-1 cells transfected with pcDNA-HBc or pcDNA-HBx were extracted to detect the translocation of c-Ets-2 by Western blotting.

**Expression and Translocation of c-Ets-2 Are Detected in THP-1 Cells with Inhibitor of MAPK Signal Pathway by Western Blotting**—The THP-1 cells transfected with pcDNA-HBc or pcDNA-HBx for 1 h were incubated in the presence or absence of 20 μM PD098059 (a specific ERK inhibitor), 20 μM SB203580 (a specific p38 MAPK inhibitor), or 10 μM SP600125 (a specific JNK inhibitor) for 40 h at 37 °C, 5% CO₂. Then cell lysates, cytoplasm, and nucleoplasm were obtained, and Western blotting was performed to detect the expression and translocation of c-Ets-2 using anti-c-Ets-2-specific antibody. Three inhibitors were purchased from BIOSOURCE.

**Construction of ERK and JNK shRNA Plasmids**—The interference shRNA plasmids for ERK and JNK were constructed using retroviral vector pMSCVneo, respectively. The sequences of target genes were as follows: ERK shRNA, 5'-AAGCATTATGACGCTACGGAGGCGGAGGAACATATCAGGCTCCTTCT-3'; JNK shRNA, 5'-AAACGACAGCTACGGAGGCGGAGGAACATATCAGGCTCCTTCT-3'; JNK shRNA, 5'-AAACGACAGCTACGGAGGCGGAGGAACATATCAGGCTCCTTCT-3'. The candidate regulatory region spanning 712 to 568 of the hFGL2 promoter was analyzed using the TFSEARCH program and the TRANSFAC data base.

**FIGURE 3.** Detection of cis-elements on hFGL2 promoter in response to HBc and HBx protein. A, relative luciferase activity of 5' truncations of hFGL2 promoter constructs in response to HBc and HBx protein in CHO cells and HepG2 cells. Left schematic drawing represents the promoter sequence of hFGL2 promoter/luciferase report constructs (relative to the transcriptional starting site). The numbers give the 5'-ending and 3'-ending nucleotide of each construct. B, location of the potential cis-elements of the hFGL2 promoter. Bold and underlined sequences indicated cis-element sites, the binding sites of potential host transcription factors involved in the transcription of the hFGL2 gene in response to HBV proteins. Boxed sequence indicated overlapped cis-element. The candidate regulatory region spanning 712 to 568 of the hFGL2 promoter was analyzed using the TFSEARCH program and the TRANSFAC data base.
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CTT GAC CAG CTG-3', and ERK control shRNA, 5'-AAT ACG GTC CAT TGA GCC ATT-3'; JNK shRNA, 5'-AAG CAC TTT CAT TCT GCT GGA-3', and JNK control shRNA, 5'-AAT TGC CAG CTG GTT CCA TCA TCA-3'. Mouse U6 promoter was amplified from mouse genomic DNA. Then the cloned U6 promoter was inserted into pMSCVneo plasmid upstream of the hairpin sequences of the target gene to form shRNA interfering plasmids, respectively. The sequences for ERK, JNK, and their control shRNA plasmids were all verified correct. THP-1 cells were transfected with HBc or HBx for 24 h and then transfected with ERK or JNK shRNA for 0, 3, 6, 9, 12, and 15 h. Cells lysates were then obtained to detect the inhibitory effect of ERK and JNK shRNA plasmids on their target gene expression with Western blotting assay.

EMSAs—To detect the effect of MAPK signal pathway on binding of transcription factor c-Ets-2 and hFGL2 promoter, THP-1 cells were divided into various groups. 1 μg of Hbc or HBx protein expression plasmid and 1 μg of ERK or JNK shRNA were mixed and cotransfected into THP-1 cells for 40 h based on the transfection manuals. Other THP-1 cells were transfected with 1 μg of HBc or HBx protein expression plasmid for 1 h and then were incubated in the presence of 20 μM PD098059 or 10 μM SP600125 for 40 h at 37 °C, 5% CO₂. Nuclear proteins were extracted from all groups, and EMSAs were performed to detect the binding of transcription factor c-Ets-2 to hFGL2 promoter.

Relative Luciferase Assay—To evaluate the effect of MAPK signal pathway on induction of hFGL2 gene, 1 μg of hFGL2 luciferase report plasmid, 1 μg of Hbc or HBx protein expression plasmid, 0.5 μg of β-galactosidase DNA, and 1 μg of ERK or JNK shRNA were mixed and cotransfected into THP-1 cells for 40 h based on the transfection manuals. Other THP-1 cells was cotransfected with 1 μg of hFGL2 luciferase report plasmid, 1 μg of Hbc or HBx protein expression plasmid, 0.5 μg of β-galactosidase for 1 h and then were incubated in the presence of 20 μM PD098059 or 10 μM SP600125 for 40 h at 37 °C, 5% CO₂. Cells were then proposed to detect the relative luciferase assay.

Statistical Analysis—Data were expressed as means ± S.D. where applicable. Student's t test for unpaired samples (two-tailed) was used to analyze the data using the SPSS 12 statistical software.

RESULTS

Expression of pcDNA-HBs, pcDNA-HBc, and pcDNA-HBx Plasmids in CHO Cells—Recombinant plasmids pcDNA-HBs, pcDNA-HBc, and pcDNA-HBx to express Hbc, Hbs, and HBx protein individually were constructed and confirmed by electrophoretic identification with restriction enzyme and sequencing. CHO cells were transiently transfected with these recombinant plasmids. Using anti-Hbc, Hbs, and HBx antibody, we first examined the expression of Hbc, Hbs, and HBx protein levels by immunocytochemistry and Western blotting, respectively. The dark brown cytoplasmic staining was seen in both pcDNA-Hbc and pcDNA-HBx transfected cells by immunocytochemistry, although there was no or trace amount of staining in pcDNA3.1 empty vector transfected cells (negative control) (Fig. 1, A–D). An expected 17-kDa band consistent with the known size of the HBx protein was specifically seen by Western blotting, which confirmed the successful expression of the pcDNA-HBx plasmid (Fig. 1E).

HBc and HBx Proteins Induce Activation of hFGL2 Prothrombinase Gene—The effects of HBV proteins on the expression of hFGL2 in cultured THP-1 cells, a human monocyte leukemia cell line, was analyzed by real time fluorescence quantitative RT-PCR and Western blotting analysis (Fig. 2, A and B). Hbc and HBx protein induced a 2.5- and 2.8-fold elevation of mRNA expression of hFGL2 in THP-1 cells compared with the pcDNA3.1 empty vector. The peak of mRNA expression was 44 h post-transfection. HBs protein failed to induce elevation of mRNA expression of hFGL2 from 24 to 72 h post-transfection. The basal mRNA levels of hFGL2 in untreated THP-1 cells were detected but weak. Western blotting assay demonstrated that HBc and HBx proteins but not HBs resulted in hFGL2 protein expression in THP-1 cells. Unlike mRNA, basal expression of hFGL2 on the protein level in untreated THP-1 cells was undetectable. It is possible that the modification post-transcription results in the dissociation of mRNA expression from hFGL2 protein. To determine the mechanism of hFGL2 expression in response to HBV proteins, CHO and HepG2 cells were transfected with the hFGL2 promoter construct hfgl2p(−1334)LUC. This construct has a 1334-bp DNA sequence upstream of the transcription start site. Cotransfection of pcDNA-HBc or pcDNA-HBx with hfgl2p(−1334)LUC resulted in a significant increase in relative luciferase activity with an increase of 5.4- and 6-fold in CHO cells and 8.7- and 11-fold increase, respectively, in HepG2 cells when compared with pcDNA3.1 empty vector cotransfected cells (Fig. 2C). There was no change in relative luciferase activity when pcDNA-HBs was cotransfected with hfgl2p(−1334)LUC in either CHO or HepG2 cells. These
results suggest that HBc and HBx proteins but not HBs protein induce transcription of hFGL2.

Mapping of the hFGL2 Promoter in Response to Viral Proteins—To characterize the region within the hFGL2 promoter that is responsive to HBc and/or HBx protein, constructs containing progressive hFGL2 promoter deletions of the −1334-bp fragment were cotransfected with either HBc or HBx expression plasmid, respectively. Similar levels of luciferase activity were detected in cells transfected with reporter gene constructs that contain 1334, 998, 817, or 712 bp of sequence upstream of the transcription start site (Fig. 3A). In contrast, luciferase activity in cells transfected with the reporter constructs that contain 568, 467, and 243 bp of sequences upstream of the transcription start site were not statistically different from basal levels measured in cells transfected with the empty vector pGL2-Basic. Thus it appears that a relatively compact region of the hFGL2 gene that spans −712 to −568 contains essential regulatory element(s) required for promoter activity in response to HBc and HBx. Using bioinformatics software Transcription Element Search System (available on line), five putative binding sites for transcription factors were identified within this region (Fig. 3B), including a tinman homeodomain factor (Nkx-2, −688 to −681), lymphocyte enhancer factor-1 (LEF-1)/c-Ets-2 (−627 to −620) (these two cis-elements were overlapped in hFGL2 promoter region), heat shock factor (HSTF, −618 to −614), a sex-determining region Y gene product (SRY, −595 to −589), and an ectopic viral integration site 1 encoded factor (Evi-1, −591 to −581)-binding site. The presence of functional competitors that included LEF-1/c-Ets-2 cis-elements or nonspecific DNA. Supershift assays were performed with nuclear extracts from THP-1 cells transfected with HBc (A), HBx (B), expressing plasmid or nontransfection (C) were incubated with a 32P-labeled probe, LEF-1/cEts-hfgl2, in −665/−617 hFGL2 promoter in the presence or absence of a 100-fold molar excess of cold

FIGURE 5. Transcription factor c-Ets-2 binds to its cognate site in the hFGL2 promoter in vitro. Nuclear extracts from THP-1 cells transfected with HBc expressing plasmid in Fig. 4A or HBx expressing plasmid in Fig. 4B were incubated with a 32P-labeled probe (LEF-1/cEts-hfgl2) in −665/−617 hFGL2 promoter in the presence or absence of a 100-fold molar excess of cold

FIGURE 6. ChIP assays demonstrated that c-Ets-2 binds to its cognate site in the hFGL2 promoter in vivo. hFGL2 proximal promoter DNA pulled down was analyzed by quantitative real time PCR as described under “Materials and Methods.” A, lanes 1–3 represent THP-1 cells transfected with pcDNA-HBc, pcDNA-HBx, and pcDNA-HBs, respectively, and lane 4 represents untreated THP-1 cells. B, data were expressed as mean fold increase ± S.D. in amount of DNA pulled down in THP-1 cells transfected with HBV protein expression plasmid relative to that in untransfected THP-1 cells. *, IP DNA by anti-c-Ets-2 antibody from THP-1 cells in response to HBc or HBx is significantly different from that of untreated cells (p < 0.05).
LEF-1-, c-Ets-2-, and HSTF-binding motifs has been suggested in previous studies to induce hFGL2 promoter activity (29–32).

**LEF/c-Ets Cis-element Accounts for the Activation of hFGL2 Gene in Response to HBc Protein and LEF/c-Ets and HSTF in Response to HBx Protein**—By site-directed mutagenesis, we were able to determine which of the five identified cis-elements were necessary for viral protein-induced transcription of the hFGL2 gene. Five mutants within the hFGL2 promoter region were then constructed according to the manufacturer’s protocol of QuickChange™ site-directed mutagenesis kit and confirmed correct by sequencing. Mutant hFGL2 promoter constructs were cotransfected with either pcDNA-HBc or pcDNA-HBx in THP-1 cells followed by relative luciferase assay. In response to HBc, LEF/c-Etsmut resulted in a 66% decrease in hFGL2 transcription activity relative to the wild type hfgl2p(−1334)LUC construct, whereas Nkx-2mut, HSFmut, SRYmut, and Evi-1mut had no statistical effect on transcription activity in response to HBc protein (Fig. 4). In contrast, in response to HBx, LEF/c-Etsmut and HSTFmut resulted in a 70 and 60% decrease of hFGL2 transcription activity relative to the wild type hfgl2p(−1334)LUC construct, whereas Nkx-2mut, SRYmut, and Evi-1mut had no effect on hFGL2 promoter activity in response to HBx protein. Thus, these results suggested that LEF/c-Ets cis-element (−627 to −620) is necessary for the activation of the hFGL2 gene in response to HBc protein and LEF/c-Ets and HSTF (−627 to −614) in response to HBx protein.

**Host Nuclear Protein c-Ets-2 Binds to Its Cis-element −625 to −620 in the hFGL2 Promoter in Response to HBc or HBx**—By mutagenesis analysis we identified three candidate cis-elements for hFGL2 regulation in response to HBc or HBx proteins. To further determine which of these cis-elements bind(s) nuclear extracts (transcription factors), EMSAs were performed using 32P-labeled LEF/cEts-hfgl2 or 32P-labeled HSTF-hfgl2 and nuclear extracts from THP-1 cells were transfected with pcDNA-HBc or pcDNA-HBx. Preliminary experiment showed binding was observed to the LEF/c-Ets-hfgl2 probe but not to the HSTF-hfgl2 probe in response to HBc or HBx. The binding of 32P-labeled LEF/cEts-hfgl2 probe with the nuclear proteins in response to HBc or HBx could be competed away by cold 100-fold molar excess unlabeled LEF/c-Ets-hfgl2 probe, but not by HSTF-hfgl2 double-stranded oligonucleotides, suggesting that cellular transcription factor(s) in nuclear extracts binds the LEF/c-Ets-hfgl2 cis-element. Yet at this point we cannot conclude that the overlapping cis-elements LEF-1 or c-Ets-2 are candidate transcription factors involved in hFGL2 gene transcription.

To further determine whether LEF-1 or c-Ets-2 bound to the hFGL2 promoter, supershift analyses were next performed. Nuclear extracts from THP-1 cells transfected with pcDNA-HBc plasmid were incubated with a 32P-labeled probe, LEF/cEts-hfgl2, in −665/−617 hFGL2 promoter in the presence or absence of polyclonal antibody against c-Ets-2. The c-Ets-2 antibody produced a specific shifted complex when added to nuclear extracts from THP-1 transfected with
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pcDNA-HBc (Fig. 5A) or pcDNA-HBx (Fig. 5B). LEF-1 antibody and a nonspecific antibody did not produce a specific shift. The protein-DNA complex in resting THP-1 cells migrated slower than that in THP-1 cells transfected with pcDNA-HBc or pcDNA-HBx, and no supershift was detected when c-Ets-2 antibody was incubated with nuclear extracts from untransfected THP-1 cells (Fig. 5C), suggesting that in resting state, the c-Ets-2 cis-element was occupied by a factor distinct from c-Ets-2 or the c-Ets-2 cis-element is conformationally changed. A \(^{32}\)P-labeled oligonucleotide probe incubated with c-Ets-2-specific antibody or irrelevant antibody in the absence of nuclear extracts failed to produce a supershift, thus verifying that the observed shifted complex was not because of an interaction between oligonucleotides and the antibody. Previously, other investigators have shown that HBc or HBx protein can directly bind to host genes and induce their transcription (33, 34). Therefore, to determine whether HBc or HBx directly bound to the regulatory domain (\(-712\) to \(-568\)), an EMSA was performed using \(^{32}\)P-labeled (\(-712/-568\))hfgl2 as probe. Nuclear extracts from THP-1 cells transfected with pcDNA-HBc or pcDNA-HBx were incubated with either polyclonal or monoclonal antibodies against HBc and HBx individually and \(^{32}\)P-labeled (\(-712/-568\))hfgl2 probe. One band was observed, which did not specifically shift in the presence or absence of anti HBc or HBx antibody (data not shown). These data suggested that hFGL2 gene transcription is not because of direct interaction between HBV proteins and the hFGL2 promoter.

To further assess the involvement of c-Ets-2 in the transcription of hFGL2 in response to HBc and HBx, we examined whether active c-Ets-2 could bind to the cis-element c-Ets-2 in the hFGL2 proximal promoter in vivo by the ChIP assay. The direct immunoprecipitation of chromatin complex by anti-c-Ets-2 confirmed that c-Ets-2 bound the c-Ets-2 cis-element within the hFGL2 promoter in THP-1 cells transfected with either pcDNA-HBc or pcDNA-HBx (Fig. 6A). A protein-DNA complex was not detected in THP-1 cells transfected with pcDNA-HBs or untreated cells. hFGL2 proximal promoter DNA pulled down was analyzed by real time quantitative PCR, which showed a 14.5- and 16.7-fold increase of c-Ets-2 with the hFGL2 proximal promoter in response to HBc and HBx relative to that in untreated cells (Fig. 6B). These results collectively demonstrate that the cis-element c-Ets-2 is functional and active in response to HBc and HBx and that hFGL2 induction by viral HBc and HBx protein is c-Ets-2-dependent.

Expression and Cellular Location of Transcriptional Factor c-Ets-2 in THP-1 Cells and CHO Cells—Because induction of hFGL2 transcription in response to viral HBc and HBx was shown to be dependent on the transcription factor c-Ets-2, we examined the expression and location of c-Ets-2 in THP-1 cells by confocal microscopy. The transcription factor c-Ets-2 was detected in the cytoplasm in untreated THP-1 cells, whereas it was translocated to the nucleus of THP-1 cells following stimulation with HBc and HBx (Fig. 7).

c-Ets-2 Short Hairpin RNA Abolishes the hFGL2 Expression in Response to HBc and HBx Protein—To confirm the involvement of c-Ets-2 in the induction of hFGL2 in response to HBc and HBx, shRNA was used to silence the endogenous c-Ets-2 expression in THP-1 cells. Transfection of c-Ets-2 shRNA resulted in significant reduction of the c-Ets-2 gene at the mRNA level as assessed by RT-PCR and real time quantitative RT-PCR when compared with either a control shRNA with a nonspecific sequence or non-shRNA transfection group (Fig. 8,
The same results were obtained at the protein level as detected by Western blotting (Fig. 8C). As shown in Fig. 8D, cotransfection of a c-Ets-2 shRNA with hfgl2p(−1334)LUC reporter plasmid significantly reduced hFGL2 promoter activity in response to HBc and HBx, whereas a control shRNA had no effect. shRNA interference of c-Ets-2 expression was able to inhibit hFGL2 gene transcription by 64.8 and 60.0% in response to HBc and HBx, respectively. These results further confirmed the critical role of c-Ets-2 in hFGL2 induction in response to HBc and HBx.

Patients—Patient characteristics are defined in Table 2. There were no statistical differences in age or gender of the patient among the three groups. All patients were HBsAg-positive, and patients with severe CHB had statistically significant increased levels of liver alanine transaminases and total bilirubin compared with either HBsAg carriers (p < 0.05, p < 0.001) or patients with mild CHB (p < 0.05, p < 0.001). Furthermore on liver biopsy, patients with severe CHB had severe inflammation and fibrosis (Metavir score F2–F4), whereas patients with mild disease had mild inflammation and minimal fibrosis (Metavir score F0–F1). Biopsies from HBsAg carriers were near normal. Levels of HBV DNA were near equivalent in patients with mild and severe CHB but markedly increased compared with carriers (p < 0.05).

Increased c-Ets-2 Protein Expression in Chronic Active Hepatitis B—To address the relevance of the transcription factor c-Ets-2 in HBV infection, we studied patients with CHB. We have previously reported the coexpression of hFGL2 mRNA transcripts and fibrin in the livers of three patients with fulminant hepatic failure because of HBV infection and 23 patients with severe CHB (16). In this study, 22 patients with biochemical, histological, and clinical evidence of severe chronic viral hepatitis B, 14 patients with mild chronic viral hepatitis B,
and 9 HBsAg carriers were studied for c-Ets-2 expression in PBMC. The characteristics of the patients are noted in Table 2. Of note, HBsAg, anti-HbcAg, and HBV DNA load did not correlate with expression of c-Ets-2. Mean levels of serum alanine transaminase ALT and total bilirubin were significantly higher in patients with severe CHB in comparison with patients with mild CHB. There was increasing c-Ets-2 expression both in the cytoplasm and nucleus of PBMC in patients with severe CHB, whereas only basal or weak c-Ets-2 expression in cytoplasm in patients with mild CHB or healthy controls (Fig. 9, A and B). These results were consistent with what was found from confocal microscopy in THP-1 cells in response to HBc and HBx in vitro. Mean fluorescence intensity of c-Ets-2 from data generated by flow cytometry showed that there was a significant increase in c-Ets-2 level in patients with severe CHB when compared with patients with mild CHB (p < 0.05), HBsAg carriers (p < 0.05), or healthy control (p < 0.001) and in patients with mild CHB when compared with healthy control (p < 0.05), demonstrating the association of c-Ets-2 expression level and severity of HBV infection (Fig. 9C). We also detect the increased immunoreactivity of c-Ets-2 in liver tissue in patients with severe CHB comparing with the mild CHB patients and healthy controls (Fig. 9D).

Activated JNK and ERK Signal Pathway in Patients with Severe CHB—To determine the signal transduction pathway upstream the c-Ets-2 transcription factor in patients with severe CHB, we examined the level of phosphorylation activation of MAPK signal pathway by Western blotting using phosphospecific antibodies (Fig. 10A). Densitometry of phosphorylation shown JNK and ERK were strongly phosphorylated in patients with severe CHB compared with mild CHB and healthy controls, and there was no significant change in the level of p38 MAPK phosphorylation between patients with mild or severe CHB or healthy controls (Fig. 10B).

Activation of c-Ets-2 and Induction of hFGL2 Gene Are Dependent on Phosphorylated MAPK Signal Pathways—As activated JNK and ERK signal pathway in patients with severe CHB were detected in comparison with patients with mild CHB, whether the MAPKs play a role in the induction of hFGL2 was further investigated. THP-1 cells were cotransfected with pcDNA-HBc or pcDNA-HBx for 0, 12, 24, 36, and 48 h, and the phosphorylated active form or total ERK, JNK, and p38 MAPKs were examined with Western blotting. The amounts of phosphorylated ERK in response to HBc appeared at 12 h, significantly increased to reach a maximum at 36 h, and then remained at the high levels at 48 h (Fig. 11A). On the other hand, the amounts of phosphorylated JNK in response to HBx occurred similarly at 12 h, significantly increased to reach a maximum at 36 h, then remained at the same levels at 48 h. p38 MAPK signal pathway was also activated but did not change with the time course of transfection suggesting that HBc and HBx did not change the active form of p38 MAPK. The expression of c-Ets-2 increased at 24 h and reached a maximum at 24–36 h. We found that hFGL2 gene occurred at 24 h and reached a maximum at 36–48 h in accordance with the activation of MAPKs and expression of c-Ets-2.

It has been reported that the MAPK signaling pathway is critical to transcriptional activity of c-Ets-2 (35, 36). To determine whether the MAPK signal pathway was involved in HBc- or HBx-induced c-Ets-2 expression and translaction, we examined the effect of MAPK inhibitors on expression and translocation of c-Ets-2. The THP-1 cells were cotransfected with HBV expression plasmids, and the amount of nuclear translocation of c-Ets-2 was determined by Western blotting analysis of the nucleoplasm and cytoplasm. Increased nuclear translocation of c-Ets-2 was observed at 12 h after cotransfection with HBc or HBx plasmids in THP-1 cells (Fig. 11B). In contrast, nuclear translocation of c-Ets-2 had not occurred in response to HBs. The data were consistent with the results of confocal microscopy in Fig. 7. Treatment with the JNK inhibitor SP600125 but not ERK inhibitor PD098059 or p38 MAPK inhibitor SB203580 inhibited the expression and translocation of c-Ets-2 in response to HBx protein, whereas the ERK inhibitor PD098059 but not the p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125 inhibited the expression and translocation of c-Ets-2 in response to HBc protein (Fig. 11C), confirming that the ERK and JNK MAPK signal pathways are involved in the expression of c-Ets-2 in response to HBc and HBx proteins, respectively. The semiquantitative analysis of the densitometry of expression of c-Ets-2 under MAPK...
inhibitors by Western blotting from total cell lysates of THP-1 cells transfected with HBc or HBx is shown on supplemental Fig. 1.

To investigate the contribution of the MAPK signal pathway in the DNA binding activity of transcription factor c-Ets-2 to hFGL2 promoter, the shRNA plasmids for ERK and JNK were constructed. Using specific phosphospecific antibodies, Western blotting assay was applied to detect the ERK and JNK level post-transfection of their corresponding ERK and JNK shRNA plasmids in THP-1 in response to HBc or HBx. The activation of ERK in response to HBc and JNK in response to HBx decreased significantly and disappeared at 15 h after transfection with ERK or JNK shRNA plasmids, demonstrating that both the ERK and JNK shRNA plasmids were effective on the interference of ERK and JNK expression (Fig. 11D). Then EMSAs were performed to detect the DNA binding using 32P-labeled LEF/cEts-hfgl2 and nuclear extracts from THP-1 cells cotransfected with pcDNA-HBc or pcDNA-HBx and ERK or JNK shRNA plasmids (Fig. 11E).

Similar to what has been shown in Fig. 5C, the protein-DNA complex in resting THP-1 cells migrated slower than that in THP-1 cells transfected with pcDNA-HBc or pcDNA-HBx. In response to HBc, treatment with ERK inhibitor or interference of ERK signal pathway shifts the band to the position in resting cells. In response to HBx, treatment with JNK inhibitor or interference of JNK signal pathway shifts the band to the position in resting cells. The results of EMSA further confirmed the obvious inhibition of c-Ets-2 DNA binding to hFGL2 promoter by ERK shRNA in response to HBc. In addition, the significant inhibition of c-Ets-2 and hFGL2 DNA binding by JNK shRNA in response to HBx was also detected. These data confirmed that the activation of c-Ets-2 DNA binding activity is mediated through MAPK signal pathways.

To examine the effect of activated MAPK signal pathway on the induction of hFGL2 gene, we detect the hFGL2 relative luciferase activity by using JNK or ERK shRNA and JNK or ERK inhibitors as described under “Materials and Methods” (Fig. 11F). Cotransfection of an ERK shRNA with hfgl2p(-1334)LUC reporter plasmid significantly re-
DISCUSSION

A number of studies have suggested that FGL2 plays a pivotal role in the pathogenesis of both experimental and human severe viral hepatitis (15–19). This study was designed to examine viral and host factors involved in transcription of hFGL2 in response to HBV infection. First we show that HBV encoding proteins HBc or HBx, but not HBs, induce transcription of hFGL2. Second, we show that the Ets family member c-Ets-2 binds to its cognate cis-element in the hFGL2 promoter and is responsible for hFGL2 gene transcription in response to HBV proteins. Third, we demonstrate that the c-Ets-2 protein and the upstream ERK and JNK signal pathway mediators were involved in expression of c-Ets-2 in response to HBc and HBx proteins, respectively. Thus, we conclude that HBc and HBx initiates the transcription of hFGL2 gene through c-Ets-2 dependent on the ERK and JNK signal pathways in response to either HBc or HBx proteins.

Using a set of parental and recombinant murine hepatitis virus strains, we previously reported that the nucleocapsid protein induced transcription of the novel mFgl2 prothrombinase gene through the transcription factor, HNF4α, in strains of mice that develop fulminant hepatitis following MHV-3 infection (20, 25). In this study, although we were able to show HBV viral protein c and x induced cEts-2 nuclear translocation in the THP-1 cell line, not all patients were HbeAg-positive in clinical samples suggesting an alternative mechanism was involved in activating c-Ets-2 that is not directly dependent upon these proteins. Previous data also reported that IFN-γ, but not IFN-α, TNF-α, or IL-1 induced mFgl2 mRNA and protein expression in both isolated peritoneal macrophages and the RAW 264.7 macrophage cell lines (24). Collectively these data suggested that there were at least two pathways, one initiated by viral proteins and a second initiated by cytokines, including TNF-α and IFN-γ, involved in mFgl2 gene transcription and subsequent protein expression in vivo. These two pathways may occur simultaneously or in sequence. Recent reports have shown following treatment with a combination of IFN-α and the nucleoside analog Ribavirin, in patients who develop a sustained anti-viral response, hFGL2 transcripts were lost (37).

Studies suggest that the HCV core protein, which is functionally analogous to the HBc or HBx, induces hFGL2 gene transcription activity as measured by an increase in a luciferase reporter gene (37, 38). We and others have demonstrated viral proteins are potent inducers of host gene expression (3–9, 20). Taken together with our previous studies, the data suggest that viral proteins and cytokines may play a synergic role in the activation of hFGL2 prothrombinase gene resulting in fibrin deposition, disturbances of microcirculation, and finally hepatocyte necrosis in patients with fulminant hepatitis or severe CHB (16, 17, 22, 23, 39, 40).

The 21-kDa HBc protein is involved in a number of important functions, including host gene regulation. Several groups have suggested the possibility of HBc as a gene regulatory protein, including the appearance of HBc in the nucleus, the presence of nucleic acid-binding motifs, nuclear localization signals, and phosphorylation sites at the serine residues of the C-terminal SPRRR motif (41). Other investigators also reported that HBc is a potent inducer of IL-18 (4) and stimulates IL-10 secretion by both T cells and monocyes from patients with CHB virus (42). HBc represses the transcription of p53 in human liver cells (8). HBx is a multifunctional protein that is known to affect gene transcription, intracellular signal transduction, genotoxic stress response, protein degradation, cell cycle control, apoptotic cell death, and carcinogenesis (3, 5–7, 9). A number of cis-elements have been reported to interact with the HBx protein and have been defined as X-responsive elements, including AP-1, AP-2, NFkB, SRF, BP, Ets, ATF1, and cAMP-response element-binding protein (43–45). Although nuclear localization signals were found in both HBc and HBx, studies also reported that viral protein could regulate host genes dependent on transcription factor(s) and signal pathway factors with the absence of nuclear localization of viral protein (5, 46). Chung et al. (5) reported that HBx protein-induced matrix metalloproteinase-9 gene activation was dependent on the transcription factor NF-κB and AP-1 and ERK and PI3K/AKT signal pathway in the absence of HBx protein nucleoli localization. Chung et al. think that stimulation of ERK and PI3K/AKT signal pathways by HBx leads to the activation of NF-κB and AP-1 transcription factor. Then translocation of NF-κB and AP-1 activated by HBx into the nucleus results in an increase of MMP-9 expression. In our study we found that HBc was expressed mainly in cytoplasm, and HBx as well as HBc protein are able to induce transcription of hFGL2 through the JNK and ERK signal pathway. By EMSA we showed that neither HBc nor HBx directly bind to hFGL2 cis-elements. By a mutation assay in THP-1 cells, we showed that LEF-1/c-Etsmut
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resulted in a 66 and 70% decrease of hFGL2 transcription activity relevant to the wild type hfgl2p(-1334)LUC construct, respectively, in response to HBc and HBx proteins, supporting the involvement of LEF/c-Ets. Further study, including mapping and mutagenesis assay, will be carried out to illuminate the distinct sequence in the HBc and HBx gene, which was responsible for the hFGL2 transcription.

By confocal microscopy, c-Ets-2 transcription factor was observed to translocate into the nuclei of THP-1 cells when transfected with either a HBc or HBx expression plasmid, which provides a mechanistic basis for the transcription factor to regulate the expression of host genes. Using EMSA and ChIP methods, we have further shown that the transcription factor c-Ets-2 but not LEF or HSTF binds its cognate cis-element and enhances hFGL2 gene transcription, arguing for the critical role of c-Ets-2 in transcription of hFGL2 in response to HBV viral proteins. When the c-Ets-2 expression was inhibited by c-Ets-2 shRNA, transcriptional activity of the hFGL2 promoter in response to HBc and HBx was decreased by 64.8 and 60%, respectively. This assay further supports a critical role for c-Ets-2 in the expression of hFGL2 in response to HBV proteins. This is consistent with a previous finding in which the interaction of PU.1, another member of the c-Ets family, with the GAS/ETS site determined the macrophage-specific induction of mFgl2 by IFN-γ.

As a human homolog of the viral oncogene, the Ets transcription factor family was originally identified in E26 avian erythrooleukemia virus, including more than 30 currently known members such as Ets-1, Ets-2, Erg-1, Erg-2, Elk, E74, Fli-1, PU.1, and PEA3 (47). Members of the Ets gene family exhibit varied patterns of tissue expression and the activity potentially to be regulated by specific intracellular signaling pathways and post-translational modification, including phosphorylation and acetylation. Ets factors are dependent on phosphorylation and acetylation and so on. In this study we demonstrated the nuclear translocation of c-Ets-2 in response to HBc and HBx proteins, indicating that c-Ets-2 was activated under the state of HBV infection. The mechanism illustrated here was different from what has been reported before, which described that HBx entered into a protein–protein complex with the cellular transcription factors cAMP-response element-binding protein and ATF-2 and altered their DNA binding specificities (57). Further studies will be undertaken to illustrate the involvement of c-Ets-2 phosphorylation in hFGL2 expression.

Furthermore, studies from patients with severe CHB have shown that c-Ets-2 protein was highly expressed in PBMC in comparison with that in patients with mild CHB and healthy controls. Although there was basal expression of MAPK in patients with mild CHB and healthy controls, there was a marked increase in phosphorylation of ERK and JNK in patients with severe CHB. The time course study of ERK and JNK activation, c-Ets-2 and hFGL2 expression in response to HBc and HBx proteins, respectively (Fig. 11A), demonstrated a correlation between ERK and JNK activation and c-Ets-2 and hFGL2 expression. Using ERK and JNK inhibitor, we were able to show the expression and translocation of c-Ets-2 in response to HBc and HBx protein were abolished (Fig. 11, B and C), suggesting the proviral involvement of ERK and JNK in c-Ets-2 translocation. Finally, the interference of JNK and ERK shRNA abolished the DNA binding activity of c-Ets-2 to the hFGL2 promoter (Fig. 11E) and subsequent hFGL2 transcription (Fig. 11F). These data confirmed that HBc and HBx mediate hFGL2 expression via activation of ERK and JNK.

In conclusion, this study has demonstrated that HBc and HBx initiates the transcription of hFGL2 through the c-Ets-2 transcription factor, dependent on the activation of ERK and JNK signal pathway, respectively. This work provides new insights into the interaction between HBV virus and the host gene hFGL2 expression, and potential therapeutic targets for controlling diseases to which hFGL2 contributed, including fulminant viral hepatitis and severe CHB, xeno- and allo-transplantation rejection, and spontaneous abortion.

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