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Antiviral Resistance Mutations Potentiate HBV Surface Antigen-Induced Transcription of *hfgl2* Prothrombinase Gene

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Abstract—Antiviral resistance mutations in the hepatitis B virus (HBV) polymerase (*pol*) gene have been demonstrated to play an important role in the progression of liver disease and the development of hepatocellular carcinoma. The HBV *pol* gene overlaps the *S* gene encoding surface antigen (HBsAg). Previous studies from our laboratory have shown that HBV core protein (HBc) and X protein (HBx), but not HBV S protein (HBs), promote *hfgl2* prothrombinase transcription. To investigate whether the nucleotide (nucleoside)-induced resistant mutations of HBs potentiate transcription of *hfgl2* prothrombinase gene, we generated two mutant HB expression constructs harboring rtM204V/sI195M or rtM204I/sW196L mutations. Two mutant expression plasmids were co-transfected with *hfgl2* promoter luciferase-reporter plasmids and β-galactosidase plasmid in CHO cells and HepG2 cells, respectively. Luciferase assay showed that the rtM204I/V mutant HBs could activate the transcription of *hfgl2* promoter compared with the wild type HBs. Site-directed mutagenesis and further experiment (co-transfection) demonstrated that transcription factor Ets translocated to its cognate *cis*-element in the *hfgl2* promoter. The results show that mutated HBs caused by antiviral drug resistance induce transcription of the *hfgl2* gene dependent on the transcription factor Ets.

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Key words: *hfgl2*, antiviral resistance, HBsAg, transcription, mutation

Hepatitis B virus (HBV) belongs to the hepadnaviridae family and causes a number of diseases including self-limiting acute hepatitis, fulminant hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. It is estimated that 400 million people worldwide are persistently infected with HBV [2, 3]. Approximately 1% of patients infected with HBV develop fulminant hepatitis [1]. In the past two decades, treatment of hepatitis B has been greatly improved with the availability of nucleoside(tide) analogs (NA) targeting the particular sites of the viral polymerase, these including lamivudine, entecavir, telbivudine, and tenofovir [4, 5]. However, emergence of drug-resistance remains a worrisome issue, which usually results in attenuated viral suppression and disease progression and may lead to significant clinical deterioration [6-8]. NA-resistant virus usually possesses characteristic amino acid substitution over the reverse transcriptase (RT) domain of the HBV polymerase gene, such as rtM204V/I and/or rtL180M for lamivudine and rtA181V/T and/or rtN236T for adefovir [9-11].

The genome of HBV is made of circular but not fully double-stranded DNA and includes four overlapping open reading frames that encode four proteins: hepatitis B core (HBc), surface (HBs), X protein (HBx), and DNA polymerase, respectively. The *S* gene is entirely overlapped by the RT domain of the polymerase gene. Although it is well known that resistance mutations also induce nonsynonymous changes in *S*, there are also several reports showing these might affect HBsAg protein conformation and its antigenicity. Torresi and colleagues reported that the reductions in the reactivity of HBsAg with vaccine-induced antibody could be caused by common lamivudine resistance mutations rtM204V/sI195M,

Abbreviations: fgl2, fibrinogen-like protein 2; HBc, hepatitis B virus core protein; HBs, hepatitis B virus S protein; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; NA, nucleoside(tide) analogs; RT, reverse transcriptase; YMDD, tyrosine-methionine-aspartate-aspartate.

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rtM204I/sW196S, rtM204I/sW196L, and rtM204V/sI195M+rtV173L/sE164D [12]. In addition, they also observed this corresponded with lamivudine resistance compensatory mutations. Mutations in the *S* gene also induced changes in the polymerase [13].

Fibrinogen-like protein 2 (fgl2)/fibroleukin, which belongs to the fibrinogen protein superfamily, has a serine protease activity and is capable of directly cleaving prothrombin to thrombin in the absence of factor VII or factor X [14, 15]. Previous studies demonstrated that mouse *fgl2* (*mfgl2*)/human *fgl2* (*hfgl2*) selective expression in the liver played a critical role in the pathogenesis of MHV-3-induced fulminant hepatitis, HBV-induced fulminant hepatitis and severe chronic hepatitis B [16-20]. High expression of *mfgl2* and *hfgl2* result in intravascular fibrin deposition within the liver, culminating in widespread hepatocyte necrosis, which correlates highly with disease severity [17, 21, 22]. Neutralizing mAb of fgl2 and genetic interference of the *mfgl2* gene prevent hepatic fibrin deposition, liver cell necrosis, and the lethality of MHV-3 infection [23]. The *fgl2* gene was also demonstrated to be involved in pathogenesis of experimental and human allograft rejection [24], spontaneous abortion, chronic obstructive pulmonary disease [25], chronic hepatitis C virus infection [26], and severe acute respiratory syndrome [27].

Our previous study demonstrated that HBc and HBx but not HBs initiate the transcription of the *hfgl2* gene [19]. In the current study we performed a series of site-directed mutagenesis experiments, inserting polymerase resistance mutations into eukaryotic expression plasmid pcDNA-HBs to characterize whether it exerted an effect on the expression of *hfgl2*.

MATERIALS AND METHODS

Construction of eukaryotic expression plasmids pcDNA-HBs. HBV DNA was isolated from serum of a patient with HBV-DNA-positive. *S* gene was amplified by polymerase chain reaction (PCR) using HBV DNA as template with primers as follows: forward, 5′-AAC ATG GAC AAC ATC ACA TC-3′, and S2 (reverse), 5′-CAA CTG CCA ATG ACA TAA CC-3′. The HBs gene was cloned into prokaryotic expression vector PCR2.1 (Invitrogen, USA) and then subcloned into eukaryotic expression vector pcDNA3.1(+) by digesting with restriction endonucleases *Hind*III and *Xho*I.

Construction of *hfgl2* promoter luciferase report plasmid. The *hfgl2* luciferase report plasmids were constructed as described previously [18]. Briefly, a proximate 1.3 kb DNA fragment containing the entire *hfgl2* promoter was released from a subclone pBluescript-m166 (p166) of human genome P1 plasmid (Genome System Inc., USA) with *Eco*RV and *Sal*I. Then the fragment was cloned into the luciferase reporter PGL2-basic (Promega, USA) to obtain the report plasmid hfgl2p(−1334)LUC. Sequential 5′-deletion constructs of the 5′-flanking region of *hfgl2* were generated by PCR using hfgl2p(−1334)LUC as a template and primers as follows: 5′-CTT ATG TCT TTC CTG CCT TC-3′ for hfgl2p(−998)LUC; 5′-GGC AAG AGA AGT TCA GGA C-3′ for hfgl2p(−817)LUC; 5′-GCA GCT ACT GGT TTT GAT G-3′ for hfgl2p(−712)LUC; 5′-TGA AGC AAA AGT 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 TGT G-3′ for hfglp2(−243)LUC. The common downstream primer used was 5′-TTC GCC CAT CTT TAC AGT G-3′. The series of PCR products were cloned into PCR2.1 cloning vector and subcloned into the *Hind*III/*Xho*I sites of PGL2-basic vector. The nucleotide sequence of the DNA fragments inserted into the luciferase vector was verified by sequencing.

Site-directed mutagenesis experiment. Constructs bearing mutant variants of the HBV *S* gene and *hfgl2* promoter were obtained by PCR using the wild type pcDNA3.1-HBs and hfgl2p(−1334)LUC as template, respectively, according to the manufacturer's protocol in the Quick-Change™ site-directed mutagenesis kit (Stratagene, USA). Primer sets used in generating site-specific mutant promoters were designed as instructed by the manufacturer and listed in the table. The boldface and underlined nucleotides in the table indicate mutated sequences. PCR cycling parameters were 95°C and 30 sec, 65°C and 1 min, and 68°C and 12 min for 20 cycles. Amplified mixtures incubated (37°C, 1 h) with *Dpn*I were used to transform XL1-Blue supercompetent cells. Finally, we constructed two single-nucleotide sequence variations in the primary structure of (wt)pcDNA-HBs. Agarose gel electrophoresis showed that (wt)pcDNA-HBs, (M1)pcDNA-HBs, and (M2)pcDNA-HBs could be digested by *Hind*III and *Xho*I restriction enzyme with a 747-bp fragment after 1 h digestion. The orientation and gene sequences of these three plasmids were confirmed by DNA sequencing. The (M1)pcDNA-HBs and (M2)pcDNA-HBs harbored the rtM204V/sI195M and rtM204I/sW196S mutations, respectively.

Cell culture. Chinese hamster ovary (CHO) cell and the human liver hepatoblastoma cell line HepG2 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. All cells were grown at 37°C in a 5% CO_2 incubator.

Transient transfection. Cells were seeded in 6-well plates at density of $5 \cdot 10^5$ cells per well for HepG2 before transfection. Transfection was accomplished with Lipofectamine 2000 reagent (Invitrogen) for CHO and HepG2 cells and Lipofectamine LTX (Invitrogen) for THP-1 cells using the manufacturer's protocol. In each experiment, 1 μg of expressing plasmid wild-type pcDNA-HBs (wt) or rtM204V/sI195M pcDNA-HBs

Primer pairs used for mutagenesis experiment

Mutant name	Sequence change	Sense primer (5′ to 3′)/antisense primer (5′ to 3′)
(M1)pcDNA-HBs	**A**TG to **G**TG	CCCACTGTTTGGCTTTCAGTTAT**G**TGGATGATG TGGTTTTG/CAAAACCACATCATCCA**C**ATAACT GAAAGCCAAACAGTGGG
(M2)pcDNA-HBs	ATG to AT**T**	CTGTTTGGCTTTCAGTTATATT**T**GATGATGT GGTTTTGGGG/CCCCAAAACCACATCATC AATAT**A**ACTGAAAGCCAAACAG
Nkx-2mut	**AATTAT** to **GCCGCG**	CAGCTACTGGTTTTGATGAAAGAC**GCCGCG** GTC CTTTTAAATGGGTCTTAGAC/GTCTAAGACCCA TTTAAAAGGAC**CGCGGC**GTCTTTCATCAAAACC AGTAGCTG
Etsmut	**AGGA** to **CACG**	CACTATGCTACGGACAA**CACG**ATA GAA AGT AGC ACTTTTTTCTCCACTAG/CTAGTGGAGAAAAAA GTGCTACTTTCTAT**CGTG**TTGTCCGTAGCATAGTG
HSTFmut	**AGA** to **TCG**	CACTATGCTACGGACAAAGGAAT**TCG**AAGTAGC ACTTTTTTCTCCACTAG/CTAGTGGAGAAAAAA GTGCTACTT**CGA**ATTCCTTTGTCCGTAGCATAGTG
SRYmut	**TAG** to **GGC**	GAAAGTAGCACTTTTTTCTCCAC**GGC**TTTTCTT CTCTTTTTCAAGTAGATGAAGC/GCTTCATCTACT TGA AAA AGA GAA GAA AA**G CC**GTGGAGA AAA AAGTGCTACTTTC
Evi-1mut	**CTT** to **GCC**	GTAGCACTTTTTTCTCCACTAGTTTT**GCC**CTCT TTTTCAAGTAGATGAAGC/GCTTCATCTACTTGA AAAAGAG**GGC**AAAACTAGTGGAGA AAA AAG TGCTAC

Note: Mutations were made to the wild-type pcDNA-HBs and the promoter sequence of *hfgl2* gene by a site-directed mutagenesis protocol as described in ''Materials and Methods''. Sense and antisense primers were designed to encode the desired mutations. The bold letters indicate the mutant sequences. All constructs generated were sequenced to confirm the orientation and to verify the sequence.

(M1) or rtM204I/sW196L pcDNA-HBs (M2) was transfected along with 1 μg of effector plasmid hfgl2p(−1334)LUC or the sequentially deleted PCR fragments of human *fgl2* promoter region (0.5 μg of β-galactosidase DNA was used as a marker for transfection efficiency). The mixture was vortexed in 100 μl of Opti-MEM medium (Invitrogen) 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 was added to bring the volume to 1 ml. One milliliter of the mixture was distributed into one well with CHO 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% CO_2 for 36-48 h.

Luciferase assay. After transfection for 44-48 h (in basal and cisplatin-induced promoter activity studies), cells were washed with 1× phosphate-buffered saline (PBS) and lysed in 250 μl/well of 1× passive lysis buffer (Promega). Luciferase and β-galactosidase activities were assayed by using the luciferase and β-galactosidase enzyme assay systems (Promega) in CHO and HepG2 cells with a Turner single tube luminometer model 20/20. 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.

Statistical analysis. Data are expressed as means ± SD where applicable. Student's *t*-test for unpaired samples (two-tailed) was used to analyze the data using the SPSS 12 statistical software.

RESULTS

Mutant HBs proteins enhanced transcription of the *hfgl2* prothrombinase gene. Luciferase reporter constructs of *hfgl2* were identified by agarose gel electrophoresis and DNA sequencing as described by Meifang Han et al. [19]. To determine whether the mutation of HBs exerts an effect on the transcription of the *hfgl2* gene, the *hfgl2* promoter construct hfgl2p(−1334)LUC was individually co-transfected with (wt)pcDNA-HBs or (M1)pcDNA-HBs or (M2)pcDNA-HBs vectors in CHO and HepG2 cells.

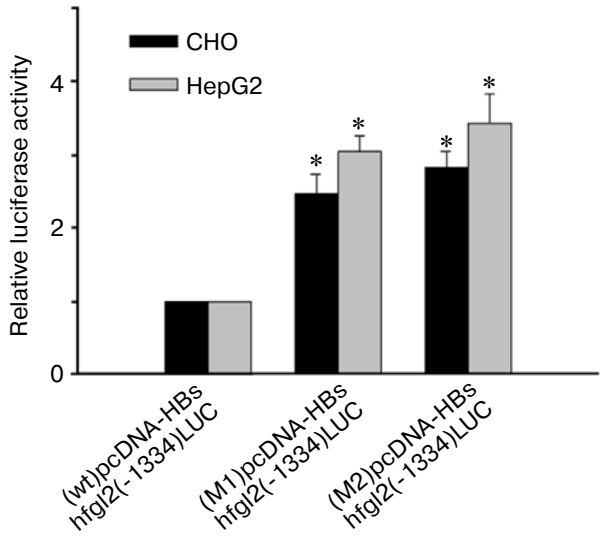

Fig. 1. Antidrug resistant mutated pcDNA-HBs induce activation of the *hfgl2* gene. rtM204V/sI195M pcDNA-HBs(M1) and rtM204I/sW196L pcDNA-HBs(M2) were co-transfected with hfgl2p(−1334)LUC in CHO and HepG2 cells for 40-44 h, and cells were harvested for measurement of luciferase activity. Values represent the means ± SD of three independent experiments. Asterisk represents $p < 0.01$ compared with cells co-transfected with wild-type pcDNA-HBs(wt).

As shown in Fig. 1, (M1)pcDNA-HBs and (M2)pcDNA-HBs induced *hfgl2* promoter activity with an average increase of 2.5- and 2.8-fold in CHO cells and 3.0- and 3.4-fold in HepG2 cells, respectively, when compared with those in cells co-transfected with wtHBs plasmid. Consistent with our previous study, there was no significant difference in relative luciferase activity when the wild-type HBs was co-transfected with hfgl2p(−1334)LUC compared with pcDNA3.1 empty group in either CHO or HepG2 cells. These results demonstrate that the mutated but not wild-type HBs enhanced the transcriptional activity of the *hfgl2* gene.

Mapping of the *hfgl2* promoter in response to mutant pcDNA-HBs. To explore the region within the *hfgl2* promoter that is responsive to mutant HBs proteins, sequential 5′-deletion constructs of *hfgl2* promotor were co-transfected with (M1)pcDNA-HBs or (M2)pcDNA-HBs expression plasmid in CHO and HepG2 cells, respectively. As shown in Fig. 2, the *hfgl2* promoter constructs that contain 998, 817, or 712 bp of sequence upstream of the transcription start site yielded similar promoter activity as the full promoter. Luciferase activity in cells transfected with the reporter constructs that contain 568, 467, and 243 bp of sequences upstream of the transcription start site significantly decreased to basal levels (Fig. 2). These results suggested the region spanning −712/−568 contained essential regulatory element(s) required for *hfgl2* promoter activity in response to mutant HBs. Using bioinformatics software Transcription Element Search System (available online), we identified five candidate transcription factor binding sites within this region including Nkx-2 (a tinman homeodomain factor), HSTF (heat-shock transcription factor), SRY (a sex-determining region *Y* gene product), Evi-1 (an ectopic viral integration site 1 encoded factor), and Ets (v-ets erythroblastosis virus E26 oncogene homolog 2) (Fig. 3a).

Ets *cis*-element accounts for activation of the *hfgl2* gene in response to mutant pcDNA-HBs. To determine which of the five identified *cis*-elements were necessary for mutated HBs-induced transcription of the *hfgl2* gene, we constructed five mutants within the *hfgl2* promoter region via site-directed mutagenesis according to the manufacturer's protocol, and the mutants were confirmed by sequencing (Fig. 3b). The mutant *hfgl2* promotor reporter plasmids were respectively co-transfected with (M1)pcDNA-HBs or (M2)pcDNA-HBs in THP-1 cell followed by relative luciferase assay. In response to (M1)pcDNA-HBs and (M2)pcDNA-HBs, Ets mutant resulted in a 47 and a 60% decrease in *hfgl2* transcription activity relative to the wild type hfgl2p(−1334)LUC construct. In contrast, Nkx-2mut, HSFmut, SRYmut, and Evi-1mut showed no statistical difference on transcription activity in response to mutated pcDNA-HBs (Fig. 4). These data suggested that Ets *cis*-element is critical in the activation of the *hfgl2* gene in response to mutated HBs.

DISCUSSION

Besides host factors, virus factors also play a significant role in the pathogenesis of severe hepatitis [19, 28, 29]. It is well-known that precore mutation at nt 1896 (G to A) and core promoter mutations at nt 1762 (A to T) and nt 1764 (G to A) are related with fulminant hepatitis [30-32]. It has been found that these mutations not only induce the reduction of HBsAg, but also enhanced replication of HBV [33-35]. In addition, several studies have shown other mutations may also be relevant with the severity of hepatitis, such as mutation in the pre-S2 start codon, mutation in the core region, insertion of nucleotides, and antidrug resistant mutations [36-39]. With the availability of oral nucleoside analog therapy, the management of hepatitis B virus has been improved in the last decade [7]. However, during long-term NA therapy, drug-resistant mutants with amino acid substitutions emerge, resulting in attenuated viral suppression and in worsening of hepatitis [11]. Lamivudine was the first nucleoside analog developed as an antiviral agent against HBV. The primary amino acid substitutions associated with the selection of lamivudine-resistance virus variants resistance is rtM204I/V, and these arise in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the polymerase, and the compensatory changes are rtL180M and rtV173L [40-43]. Reports have shown that lamivu-

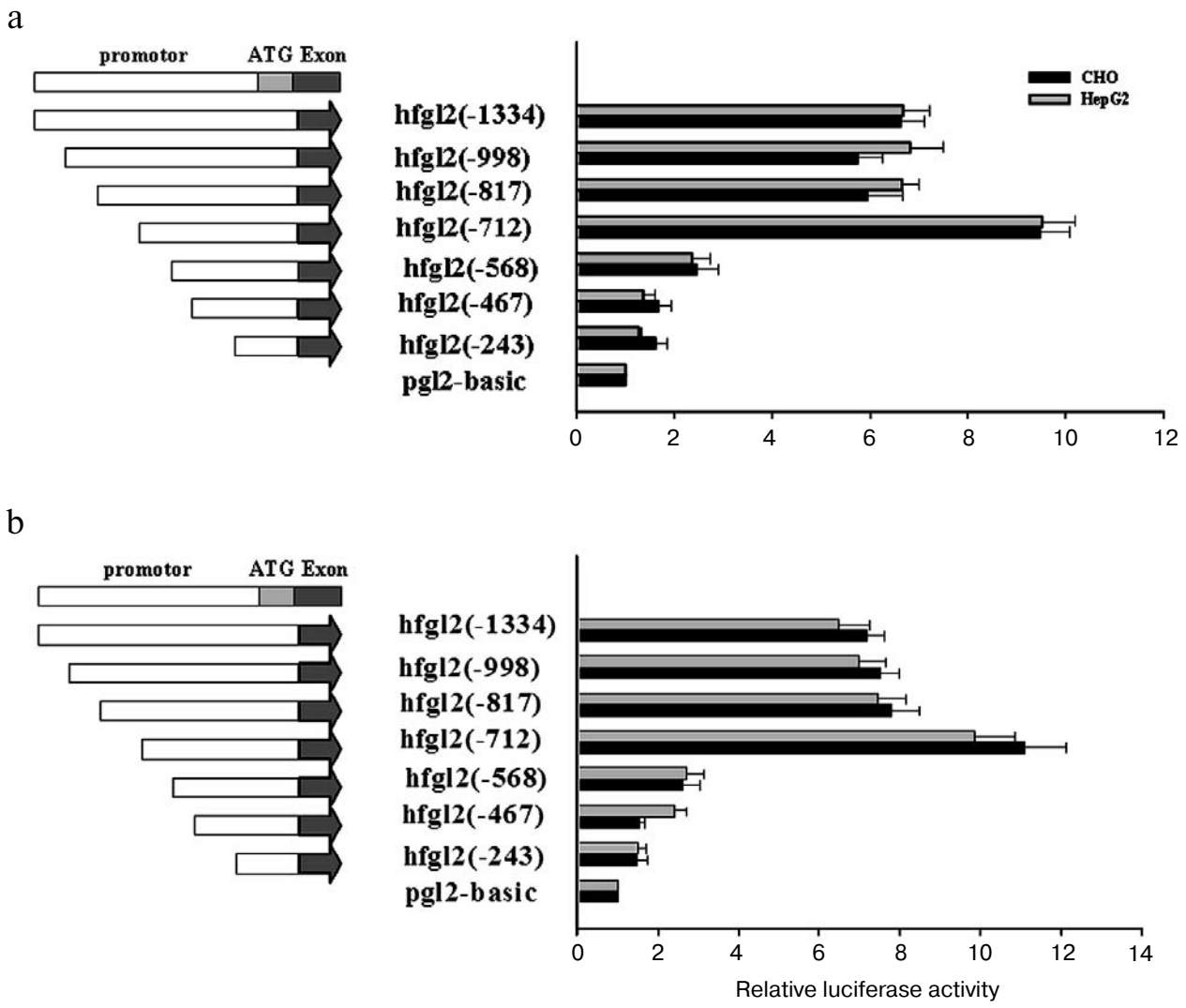

Fig. 2. Mapping of the *hfgl2* promoter in response to rtM204V/sI195M pcDNA-HBs(M1) (a) and rtM204I/sW196L pcDNA-HBs (M2) (b). The mutated pcDNA-HBs plasmid was co-transfected with progressive deletions of the *hfgl2* promoter luciferase-reporter plasmid into CHO and HepG2 cells, respectively. The cells transfected with pGL2-Basic vectors was the control group. After transfection for 40-44 h, cells were harvested for measurement of relative luciferase activity.

dine-associated mutations are related to severe hepatitis [28, 39, 44].

Previously our studies and those of many others have implicated fgl2 as playing a pivotal role in the pathogenesis of both experimental and human severe viral hepatitis as well as in human hepatocellular carcinoma [17, 45, 46]. The HBV-encoded proteins HBc and HBx were available to induce the activation of the *hfgl2* prothrombinase gene, whereas HBs protein failed to activate *hfgl2* transcription [19]. In the present study, we generated two mutated eukaryotic expression plasmids of pcDNA-HBs that contain rtM204V/sI195M and rtM204I/sW196L mutation, respectively. By co-transfection assay we defined that the *hfgl2* gene transcriptions activated by rtM204V/sI195M and rtM204I/sW196L pcDNA-HBs were significantly higher than those of wild-type pcDNA-HBs. Preliminary mapping of the *hfgl2* gene promoter, the region from −712 to −467 (relative to transcription start site) in the *hfgl2* promoter was defined to be the regulatory region responsive to mutated HBs. By mutagenesis assay, we further demonstrated that mutated HBs protein induced activation of the *hfgl2* gene dependent on transcription factor Ets. The Ets transcription factor has been reported to activate or repress the expression of genes that are involved in various biological processes, including cellular proliferation, differentiation, development, transformation, and apoptosis [47]. Earlier investigations demonstrated that Ets-1 was required for the development and function of natural regulatory T cells [48]. Endothelial-specific microRNA-126 have been

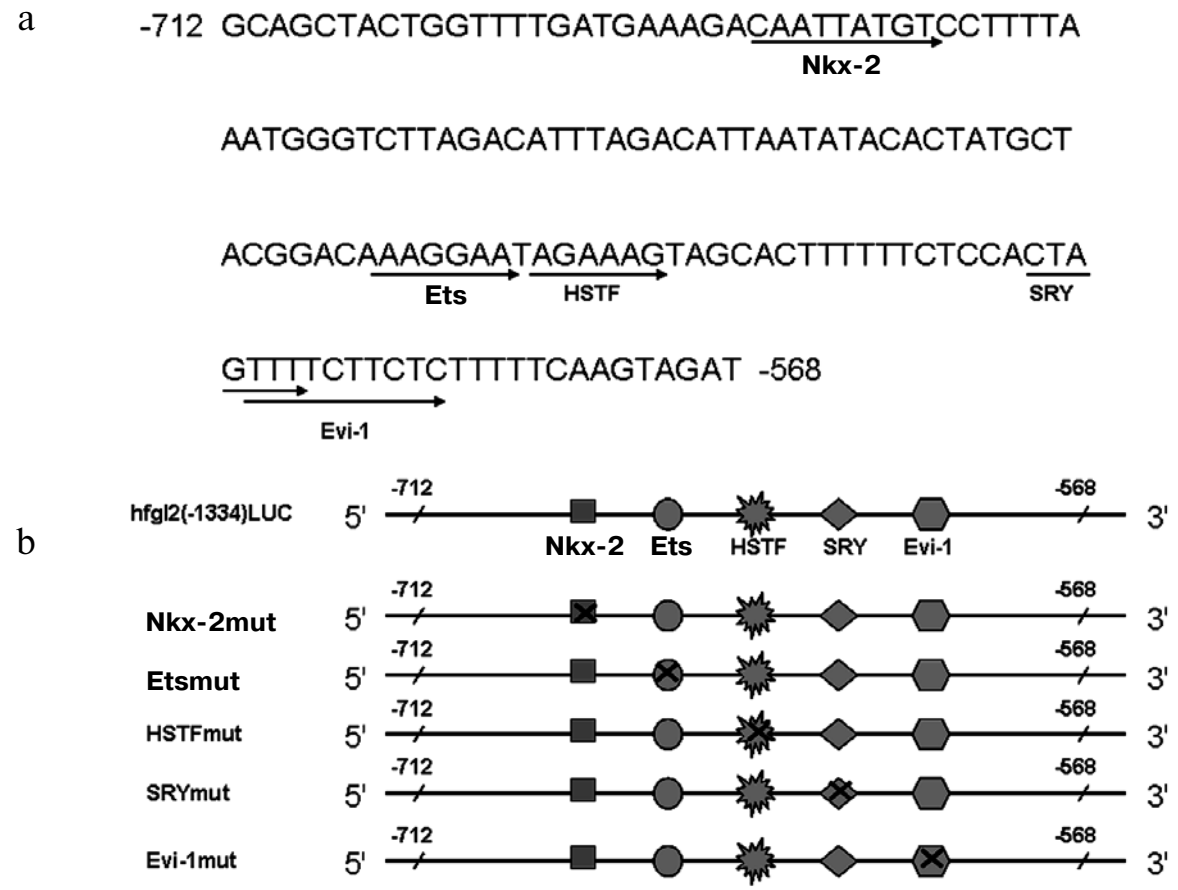

Fig. 3. Putative regulatory element analysis response to mutated pcDNA-HBs. a) Location of the potential *cis*-elements in the putative (−817 to −467) *hfgl2* promoter. Underlined sequences indicated *cis*-element sites, the binding sites of potential host transcription factors involved in the transcription of the *hfgl2* gene in response to mutated pcDNA-HBs. b) Schematic representation of the putative regulatory element *hfgl2* promoter in response to mutated pcDNA-HBs.

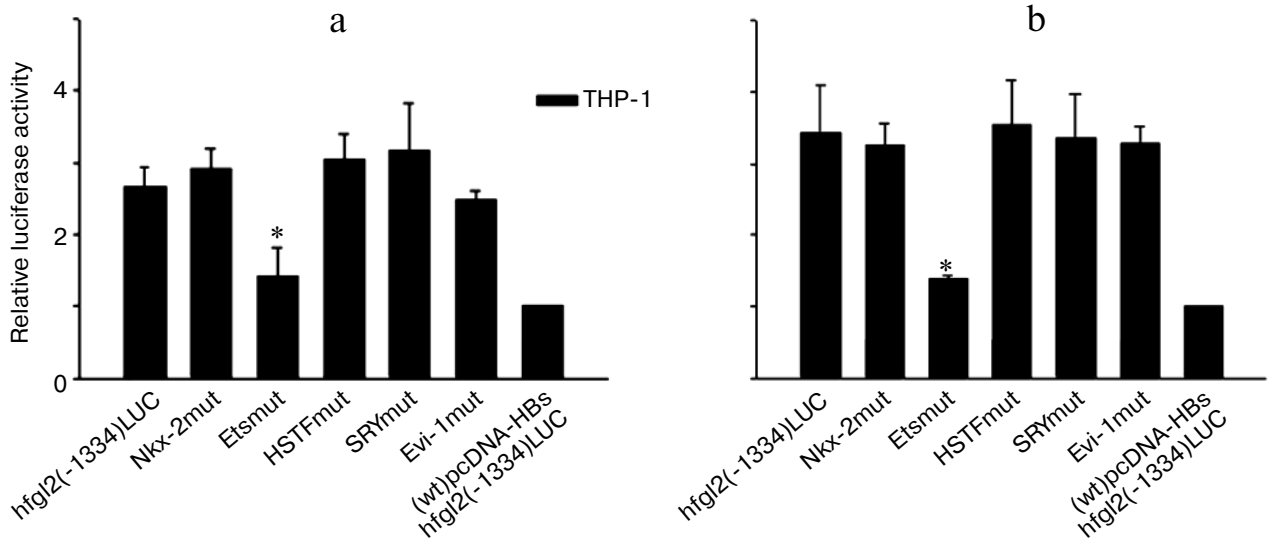

Fig. 4. Site-directed mutagenesis analysis for *cis*-elements of the *hfgl2* gene in response to mutated pcDNA-HBs. Mutation assay showed transient expression of luciferase activity induced by the *hfgl2* promotor mutant variants in response to mutated (M1)pcDNA-HBs (a) and (M2)pcDNA-HBs (b) in transfected THP-1 cells. *$p < 0.01$ compared with cells co-transfected with wild-type hfgl2p(−1334)LUC construct. All luciferase assays represent the mean ± SD of three independent experiments done in triplicate.

reported to be regulated by transcription factors Ets-1 and Ets-2 [49]. It has also been reported that Ets-1 in the orchestration of a network of molecular and phenotypic events contributes to the malignant phenotype of mammary cancer cells within their matrix environment and appears as a probable key step for breast cancer progression [50].

Since the HBV surface antigen (HBsAg) gene is entirely overlapped by the RT-gene, some lamivudine resistance RT mutations produce mutations in HBsAg that can lead to a reduced HBsAg antigenicity [51-54]. Warner and colleagues showed that the rtA181T/sW172 variant revealed a secretory defect and exerts a dominant negative effect on wild-type HBV virion secretion [55]. Sloan reported that HBsAg immunoreactivity could be altered through concomitant amino acid substitutions at codons within and downstream from the a determinant by mutations associated with resistance to NA therapy, singly or in combination with each other or antibody escape-associated mutations [56]. It is reported that HBV polymerase rtA181T/surface truncation mutant activated the transcription of *c-myc* gene promoter, which showed oncogenic potential [6]. In our study we found that the HBV polymerase YMDD/surface mutation potentiated *hfgl2* transcription via transcriptional factor Ets. These studies collectively explained in part the contribution of mutant HBV in disease progression in severe chronic hepatitis B patients.

In summary, this study has demonstrated that YMDD mutated HBs initiate the transcription of the *hfgl2* gene through Ets transcriptional factor binding to its *cis*-element and contributes to the understanding of mutant HBV-relevant disease deterioration.

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REFERENCES

1. Chen, Y., Michitaka, K., Matsubara, H., Yamamoto, K., Horiike, N., and Onji, M. (2003) *J. Hepatol.*, **38**, 84-90.
2. Maddrey, W. C. (2000) *J. Med. Virol.*, **61**, 362-366.
3. Lavanchy, D. (2004) *J. Viral Hepatol.*, **11**, 97-107.
4. Keeffe, E. B., Dieterich, D. T., Han, S. H., Jacobson, I. M., Martin, P., Schiff, E. R., and Tobias, H. (2008) *Clin. Gastroenterol. Hepatol.*, **6**, 1315-1341; quiz 1286.
5. Lok, A. S., and McMahon, B. J. (2009) *Hepatology*, **50**, 661-662.
6. Lai, M. W., and Yeh, C. T. (2008) *Antivir. Ther.*, **13**, 875-879.
7. Ghany, M. G., and Doo, E. C. (2009) *Hepatology*, **49**, S174-184.
8. Lok, A. S., Zoulim, F., Locarnini, S., Bartholomeusz, A., Ghany, M. G., Pawlotsky, J. M., Liaw, Y. F., Mizokami, M., and Kuiken, C. (2007) *Hepatology*, **46**, 254-265.
9. Osiowy, C., Villeneuve, J. P., Heathcote, E. J., Giles, E., and Borlang, J. (2006) *J. Clin. Microbiol.*, **44**, 1994-1997.
10. Bartholomeusz, A., and Locarnini, S. A. (2006) *Semin. Liver Dis.*, **26**, 162-170.
11. Hatakeyama, T., Noguchi, C., Hiraga, N., Mori, N., Tsuge, M., Imamura, M., Takahashi, S., Kawakami, Y., Fujimoto, Y., Ochi, H., Abe, H., Maekawa, T., Kawakami, H., Yatsuji, H., Aisaka, Y., Kohno, H., Aimitsu, S., and Chayama, K. (2007) *Hepatology*, **45**, 1179-1186.
12. Torresi, J., Earnest-Silveira, L., Deliyannis, G., Edgtton, K., Zhuang, H., Locarnini, S. A., Fyfe, J., Sozzi, T., and Jackson, D. C. (2002) *Virology*, **293**, 305-313.
13. Torresi, J., Earnest-Silveira, L., Civitico, G., Walters, T. E., Lewin, S. R., Fyfe, J., Locarnini, S. A., Manns, M., Trautwein, C., and Bock, T. C. (2002) *Virology*, **299**, 88-99.
14. Levy, G. A., Liu, M., Ding, J., Yuwaraj, S., Leibowitz, J., Marsden, P. A., Ning, Q., Kovalinka, A., and Phillips, M. J. (2000) *Am. J. Pathol.*, **156**, 1217-1225.
15. Yuwaraj, S., Ding, J., Liu, M., Marsden, P. A., and Levy, G. A. (2001) *Genomics*, **71**, 330-338.
16. Ding, J. W., Ning, Q., Liu, M. F., Lai, A., Peltekian, K., Fung, L., Holloway, C., Yeger, H., Phillips, M. J., and Levy, G. A. (1998) *Adv. Exp. Med. Biol.*, **440**, 609-618.
17. Marsden, P. A., Ning, Q., Fung, L. S., Luo, X., Chen, Y., Mendicino, M., Ghanekar, A., Scott, J. A., Miller, T., Chan, C. W., Chan, M. W., He, W., Gorczynski, R. M., Grant, D. R., Clark, D. A., Phillips, M. J., and Levy, G. A. (2003) *J. Clin. Invest.*, **112**, 58-66.
18. Ning, Q., Liu, M., Kongkham, P., Lai, M. M., Marsden, P. A., Tseng, J., Pereira, B., Belyavskyi, M., Leibowitz, J., Phillips, M. J., and Levy, G. (1999) *J. Biol. Chem.*, **274**, 9930-9936.
19. Han, M., Yan, W., Guo, W., Xi, D., Zhou, Y., Li, W., Gao, S., Liu, M., Levy, G., Luo, X., and Ning, Q. (2008) *J. Biol. Chem.*, **283**, 32715-32729.
20. Zhu, C. L., Yan, W. M., Zhu, F., Zhu, Y. F., Xi, D., Tian, D. Y., Levy, G., Luo, X. P., and Ning, Q. (2005) *World J. Gastroenterol.*, **11**, 6936-6940.
21. Ding, J. W., Ning, Q., Liu, M. F., Lai, A., Leibowitz, J., Peltekian, K. M., Cole, E. H., Fung, L. S., Holloway, C., Marsden, P. A., Yeger, H., Phillips, M. J., and Levy, G. A. (1997) *J. Virol.*, **71**, 9223-9230.
22. Ning, Q., Brown, D., Parodo, J., Cattral, M., Gorczynski, R., Cole, E., Fung, L., Ding, J. W., Liu, M. F., Rotstein, O., Phillips, M. J., and Levy, G. (1998) *J. Immunol.*, **160**, 3487-3493.
23. Zhu, C., Sun, Y., Luo, X., Yan, W., Xi, D., and Ning, Q. (2006) *Hum. Gene Ther.*, **17**, 589-600.
24. Ning, Q., Sun, Y., Han, M., Zhang, L., Zhu, C., Zhang, W., Guo, H., Li, J., Yan, W., Gong, F., Chen, Z., He, W., Koscik, C., Smith, R., Gorczynski, R., Levy, G., and Luo, X. (2005) *J. Immunol.*, **174**, 7403-7411.
25. Liu, Y., Xu, S., Xiao, F., Xiong, Y., Wang, X., Gao, S., Yan, W., and Ning, Q. (2010) *Biochem. Biophys. Res. Commun.*, **396**, 555-561.
26. Foerester, K., Helmy, A., Zhu, Y., Khattar, R., Adeyi, O. A., Wong, K. M., Shalev, I., Clark, D. A., Wong, P. Y., Heathcote, E. J., Phillips, M. J., Grant, D. R., Renner, E. L., Levy, G. A., and Selzner, N. (2010) *J. Hepatol.*, **53**, 608-615.
27. Han, M., Yan, W., Huang, Y., Yao, H., Wang, Z., Xi, D., Li, W., Zhou, Y., Hou, J., Luo, X., and Ning, Q. (2008) *J. Biochem.*, **144**, 51-62.
28. Bonacini, M., Kurz, A., Locarnini, S., Ayres, A., and Gibbs, C. (2002) *Gastroenterology*, **122**, 244-245.

29. Baumert, T. F., Yang, C., Schurmann, P., Kock, J., Ziegler, C., Grullich, C., Nassal, M., Liang, T. J., Blum, H. E., and von Weizsacker, F. (2005) *Hepatology*, **41**, 247-256.
30. Omata, M., Ehata, T., Yokosuka, O., Hosoda, K., and Ohto, M. (1991) *N. Engl. J. Med.*, **324**, 1699-1704.
31. Teo, E. K., Ostapowicz, G., Hussain, M., Lee, W. M., Fontana, R. J., and Lok, A. S. (2001) *Hepatology*, **33**, 972-976.
32. Rezende, R. E., Fonseca, B. A., Ramalho, L. N., Zucoloto, S., Pinho, J. R., Bertolini, D. A., and Martinelli, A. L. (2005) *J. Clin. Virol.*, **32**, 53-59.
33. Lamberts, C., Nassal, M., Velhagen, I., Zentgraf, H., and Schroder, C. H. (1993) *J. Virol.*, **67**, 3756-3762.
34. Moriyama, K., Okamoto, H., Tsuda, F., and Mayumi, M. (1996) *Virology*, **226**, 269-280.
35. Buckwold, V. E., Xu, Z., Chen, M., Yen, T. S., and Ou, J. H. (1996) *J. Virol.*, **70**, 5845-5851.
36. Ehata, T., Omata, M., Chuang, W. L., Yokosuka, O., Ito, Y., Hosoda, K., and Ohto, M. (1993) *J. Clin. Invest.*, **91**, 1206-1213.
37. Pollicino, T., Zanetti, A. R., Cacciola, I., Petit, M. A., Smedile, A., Campo, S., Sagliocca, L., Pasquali, M., Tanzi, E., Longo, G., and Raimondo, G. (1997) *Hepatology*, **26**, 495-499.
38. Pult, I., Chouard, T., Wieland, S., Klemenz, R., Yaniv, M., and Blum, H. E. (1997) *Hepatology*, **25**, 1507-1515.
39. Peters, M. G., Singer, G., Howard, T., Jacobsmeyer, S., Xiong, X., Gibbs, C. S., Lamy, P., and Murray, A. (1999) *Transplantation*, **68**, 1912-1914.
40. Bartholomew, M. M., Jansen, R. W., Jeffers, L. J., Reddy, K. R., Johnson, L. C., Bunzendahl, H., Condreay, L. D., Tzakis, A. G., Schiff, E. R., and Brown, N. A. (1997) *Lancet*, **349**, 20-22.
41. Dienstag, J. L., Schiff, E. R., Wright, T. L., Perrillo, R. P., Hann, H. W., Goodman, Z., Crowther, L., Condreay, L. D., Woessner, M., Rubin, M., and Brown, N. A. (1999) *N. Engl. J. Med.*, **341**, 1256-1263.
42. Dienstag, J. L., Schiff, E. R., Mitchell, M., Casey, D. E., Jr., Gitlin, N., Lissoos, T., Gelb, L. D., Condreay, L., Crowther, L., Rubin, M., and Brown, N. (1999) *Hepatology*, **30**, 1082-1087.
43. Allen, M. I., Deslauriers, M., Andrews, C. W., Tipples, G. A., Walters, K. A., Tyrrell, D. L., Brown, N., and Condreay, L. D. (1998) *Hepatology*, **27**, 1670-1677.
44. Bessesen, M., Ives, D., Condreay, L., Lawrence, S., and Sherman, K. E. (1999) *Clin. Infect. Dis.*, **28**, 1032-1035.
45. McGilvray, I. D., Lu, Z., Wei, A. C., Dackiw, A. P., Marshall, J. C., Kapus, A., Levy, G., and Rotstein, O. D. (1998) *J. Biol. Chem.*, **273**, 32222-32229.
46. Su, K., Chen, F., Yan, W. M., Zeng, Q. L., Xu, L., Xi, D., Pi, B., Luo, X. P., and Ning, Q. (2008) *World J. Gastroenterol.*, **14**, 5980-5989.
47. Seth, A., and Watson, D. K. (2005) *Eur. J. Cancer*, **41**, 2462-2478.
48. Mouly, E., Chemin, K., Nguyen, H. V., Chopin, M., Mesnard, L., Leite-de-Moraes, M., Burlen-Defranoux, O., Bandeira, A., and Bories, J. C. (2010) *J. Exp. Med.*, **207**, 2113-2125.
49. Harris, T. A., Yamakuchi, M., Kondo, M., Oettgen, P., and Lowenstein, C. J. (2010) *Arterioscler. Thromb. Vasc. Biol.*, **30**, 1990-1997.
50. Furlan, A., Vercamer, C., Desbiens, X., and Pourtier, A. (2008) *J. Cell Physiol.*, **215**, 782-793.
51. Locarnini, S. A. (1998) *Hepatology*, **27**, 294-297.
52. Carman, W. F. (1997) *J. Viral Hepat.*, **4** (Suppl. 1), 11-20.
53. Sheldon, J., and Soriano, V. (2008) *J. Antimicrob. Chemother.*, **61**, 766-768.
54. Chen, W. N., and Oon, C. J. (2000) *J. Clin. Microbiol.*, **38**, 2793-2794.
55. Warner, N., and Locarnini, S. (2008) *Hepatology*, **48**, 88-98.
56. Sloan, R. D., Ijaz, S., Moore, P. L., Harrison, T. J., Teo, C. G., and Tedder, R. S. (2008) *Antivir. Ther.*, **13**, 439-447.