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In vitro resistance to interferon-alpha of hepatitis B virus with basic core promoter double mutation

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Abstract

The hepatitis B virus (HBV) genome basic core promoter (BCP) modulates HBeAg secretion at the transcriptional level. In addition to pre-core mutations, variations in the BCP are related to hepatitis B e antigen (HBeAg)-negative chronic hepatitis B. HBeAg-negative chronic hepatitis B patients show a lower sustained response to interferon (IFN). The aim of this study was to determine if there is a relationship between HBV BCP mutation and sensitivity of HBV to IFN-alpha *in vitro*. BCP mutations were introduced by site-directed mutagenesis and the entire genomes of wild-type and mutant HBV were transiently transferred into Huh7 cells by calcium phosphate transfection. With or without IFN-alpha, viral products in the culture medium and viral replication intermediates in the cytoplasm were detected 3 days after transfection. The amount of hepatitis B surface antigen (HBsAg) secreted by wild-type HBV and the BCP mutant was similar, while HBeAg secreted by the mutant was decreased by 35.4%. HBV particles and replication intermediates of the BCP mutant were increased. After IFN-alpha was added, HBeAg, HBV DNA and HBV replication intermediates decreased for both the wild-type HBV (by 25.7%, 31.8%, 29.8%, respectively) and the BCP mutant (by 8.4%, 27.4%, 10.1%, respectively). These data indicate that HBV harboring the BCP double mutation has stronger replication competence and lower sensitivity to IFN-alpha than wild-type.

Keywords: Hepatitis B virus; Basic core promoter; Mutation; Interferon-alpha

1. Introduction

Chronic hepatitis B virus infection remains a major public health problem worldwide. Despite a high rate of viral clearance by immunocompetent adults and the availability of efficient vaccines, a large proportion of the world's population (400 million) is chronically infected with HBV. Chronic hepatitis B (CHB) virus infection can result in a wide spectrum of liver diseases. Current strategies for treating hepatitis B have focused on clearance of active HBV infection through suppression of viral replication. IFN-alpha and nucleos(t)ide analogs (lamivudine and adefovir) (Marcellin et al., 2003; Hadziyannis et al., 2003) have been approved for treatment by the FDA. Chronic HBV infection is predominantly treated with IFN-alpha, which results in efficient reduction of the viral load in only 20–40% of patients.

HBV is a small enveloped DNA virus, the replication of which involves reverse transcription of pregenomic RNA (Summers and Mason, 1982). The HBV genome is 3.2 kb in length and consists of partially double-stranded DNA that contains four open reading frames (ORFs) encoding core, surface, polymerase-reverse transcriptase and X gene products (Will et al., 1987). The expression of these viral gene products is regulated by four promoters that direct the generation of 3.5 kb, 2.4 kb, 2.1 kb and 0.7 kb mRNAs, respectively. The HBV core promoter regulates transcription of the 3.5 kb C mRNA/pregenome that not only serves for translation of core protein and polymerase-reverse transcriptase, but is also the template for reverse transcription (Yuh et al., 1992). Only HBeAg precursor is translated from the second core promoter transcript, precore mRNA. Because of its crucial role in the viral life cycle, naturally occurring sequence variation found in the HBV core

Abbreviations: HBeAg, hepatitis B e antigen; BCP, basic core promoter; HBV, hepatitis B virus; IFN, interferon; HBsAg, hepatitis B surface antigen; ORFs, open reading frames; nt, nucleotide; dNTPs, deoxynucleoside triphosphate; ELISA, enzyme-linked immunosorbent assay; pgRNA, pregenome RNA; CURS, core upstream regulatory sequences; NRE, negative regulatory element

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promoter of certain patients is currently the focus of intense investigation. A double mutation, A1762T and G1764A, in the core promoter has frequently been found in HBV strains isolated from patients with chronic hepatitis B (Okamoto et al., 1994; Kurosaki et al., 1996).

Although the BCP influences the clinical status of HBV infection, the role of BCP mutations in the progression of chronic hepatitis and IFN-alpha sensitivity are still unknown. This study was designed to evaluate the relationship between HBV BCP mutation and sensitivity of HBV to IFN-alpha *in vitro*.

2. Materials and methods

2.1. Amplification of the HBV genome

Serum was collected from an 11-year-old male asymptomatic carrier of HBsAg who had not received IFN-alpha or lamivudine treatment. The serum also tested positive for HBeAg and anti-HBc. The serum contained 6×10^8 copies/ml of HBV genotype C a normal ALT level (<1 ULN). Serum HBV DNA was isolated by digestion with Proteinase K followed by phenol/chloroform extraction and ethanol precipitation. The entire HBV genome (3.2 kb) was amplified with the following primers (Gunther et al., 1995): P1 (5′-CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA, 1821–1841) and P2 (5′-CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG, 1825–1806) (*Sap*I sites are underlined). PCR amplification was performed using the Expand High-Fidelity PCR System (Roche) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 40 s, 60 °C for 1 min and 68 °C for 3 min, with an increment of 5 s for each remaining cycle; and a final extension at 72 °C for 7 min.

2.2. Cloning and sequencing of the HBV genome

PCR products 3.2 kb in length were isolated and ligated into the pGEM-T easy vector (Promega) according to the Manufacturer's instructions. Ligation products were transformed into JM109 calcium chloride competent cells. Positive clones were selected and checked by digestion with *Eco*RI. The HBV genome was released from a positive clone and transfected into Huh7 cells. The most competent clone 57-1 with full length HBV was confirmed by DNA sequencing and named pGEM-T-HBV.

2.3. Generation of a BCP double mutant

Site-directed mutagenesis, using pGEM-T-HBV as the template, was used to introduce a BCP double mutation, A1762T and G1764A, into the HBV genome (Fig. 1). The following primers were used for mutagenesis: 1762/64 sense (5′-GAGGAGATTAGGTTAATGATCTTTGTACTAGGAGGC 3′) and 1762/64 anti (5′-GCCTCCTAGTACAAAGATCATTAACTAATCTCCT); PS5 sense (1260–1279, 5′-GCCGATCCATACTGCGGAAC) and SP6 promoter (5′-ATTTAGGTGACACTATAGAA) primers were also used. All mutagenesis reactions were carried out using Expand High-Fidelity PCR System (Roche).

In the first round of PCR, the1762/64 sense and SP6 promoter primers were used to acquire a 188 bp fragment, and 1762/64 anti-PS5 sense primers produced a 503 bp fragment. The two fragments were then used as templates in the second round of PCR. A 676 bp-fragment, amplified with the PS5 sense and SP6 promoter primers, was cloned into the pGEM-T easy vector. After sequencing to rule out undesired misincorporations, a *Pst*I–*Bam*HI fragment with the BCP double mutation was excised and exchanged with the corresponding wild-type *Pst*I–*Bam*HI fragment from pGEM-T-HBV. The resultant plasmid, designated pGEM-T-HBV-TA, contained the 1762T/1764A mutations.

2.4. Preparation of HBV for transfection

Bacteria strains containing the pGEM-T-HBV or pGEM-T-HBV-TA were cultured at large scale and the two plasmids were extracted and purified. The plasmids were digested with *Sca*I to destroy the vector, whose length was similar to that of the HBV genome, extracted twice with phenol:chloroform:isoamyl alcohol, and precipitated. The recovered plasmids were digested with *Sap*I overnight and separated on a 0.8% agarose gel. The 3.2 kb linear HBV monomers were purified with the QIAquick Gel Extraction Kit (Qiagen) and quantified for transfection.

2.5. Transfection of HBV DNA by calcium phosphate precipitation

Huh7 cells were plated at a density of 1.3×10^6 per 60-mm diameter petri dish (Falcon) 1 day before transfection. The medium was changed 4 h before transfection. Calcium phosphate precipitation transfection was carried out according to the Manufacturer's protocol (ProFection Mammalian Transfection System—Calcium Phosphate; Promega). Medium with or without 1000 IU ml^{-1} IFN-alpha (Interferon alfa-2b; Intron A, Schering-Plough Research Institute) was added 24 h after shock, and the culture medium and cells were harvested 3 days later. Transfection efficiency was normalized by the activity of secreted alkaline phosphatase (SEAP) as previously described (Wen et al., 1986; Berger et al., 1988; Cullen and Malim, 1992).

2.6. Detection of HBsAg, HBeAg and HBV DNA secreted into culture medium

HBsAg and HBeAg in the cell culture supernatant were assayed with commercial enzyme-linked immunosorbent assay (ELISA) kits. HBsAg was assayed with Hepanostika HBsAg Uni-Form II (Biomérieux bv, Netherlands) and RADIM (Italia) was used for HBeAg. Transfected Huh7 cell culture medium was collected 3 days after transfection and centrifuged at 14,000 rpm for 10 min at 4 °C to remove cell debris. The supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg ml^{-1} DNase I for 60 min at 37 °C to remove the remaining linear HBV monomers. HBV DNA in the cell medium was detected by fluorescent real-time quantitative PCR (commercially available assay kit, Roche Light Cycler).

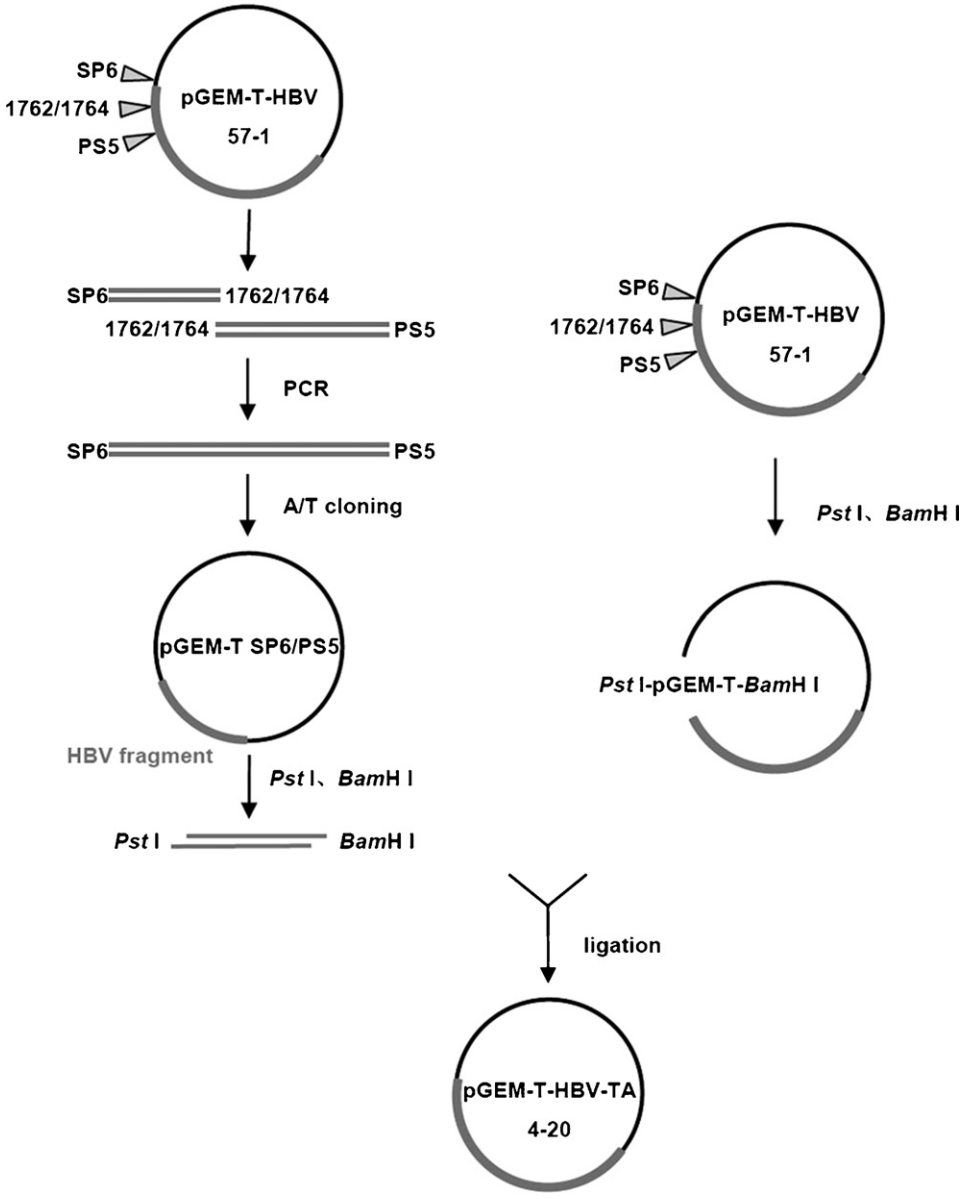

Fig. 1. Construction of plasmid containing the A1762T/G1764A mutation. The HBV BCP 1762/1764 mutation was introduced into a short PCR product by PCR site-directed mutagenesis. The PCR product replaced the corresponding fragment of the wild-type clone to create pGEM-T-HBV-TA that contains the A1762T/G1764A mutations.

2.7. Purification of HBV DNA from intracellular core particles and Southern blot analysis

Cells were washed and lysed in 0.5 ml of lysis buffer (50 mM Tris–HCl [pH 7.4], 1 mM EDTA, 1% Nonidet P-40) at 72 h after transfection. The lysates were incubated on ice for 15 min, after which nuclei were pelleted by centrifugation. The supernatant was treated with 10 mM $MgCl_2$ and 100 mg ml^{-1} of DNase I for 60 min at 37 °C, and then digested with 1% sodium dodecyl sulfate and 0.5 mg ml^{-1} Proteinase K for 1 h at 60 °C. The DNA was separated on a 1.0% agarose gel, blotted onto Magnaprobe membranes (Osmonics), and hybridized with digoxin labeled full-length HBV probe (PCR DIG Probe Synthesis Kit (Roche). The probe was purified with the QIAquick Gel Extraction Kit and quantified for hybridization. A DIG Luminescent Detection Kit (Roche) and DIG Easy Hyb (Roche) were used to perform the hybridization reaction. AP-anti-Dig (1 IU μl^{-1}) at 1:5000 and CSPD (25 mM) at 1:100 were used to detect probe on the membrane. Fluorescence signals were detected with Kodak X-ray film and quantified with Quantity One software (Biorad).

2.8. Determination of optimum IFN-alpha concentration in vitro

The optimal concentration of IFN-alpha *in vitro* was determined by adding different concentrations of IFN-alpha-2b to the culture medium. The serial concentrations of IFN-alpha used in the experiment were 62.5 IU ml^{-1}, 125 IU ml^{-1}, 250 IU ml^{-1}, 500 IU ml^{-1}, 1000 IU ml^{-1}, 2000 IU ml^{-1}, 4000 IU ml^{-1} and 8000 IU ml^{-1}. Three days after transfection, the culture medium

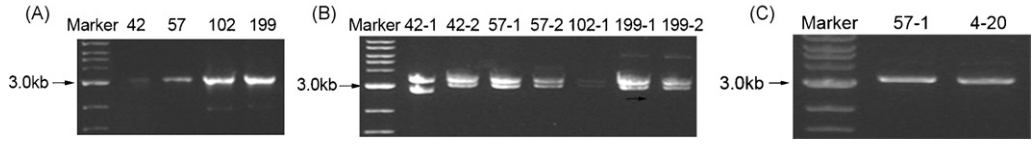

Fig. 2. Electrophoresis of HBV DNA PCR product and digested clones. (A) HBV DNA PCR product from different serum. The sample IDs are listed above the corresponding lane. (B) Electrophoresis of different clones digested with *Eco*RI. Clones 42-2, 57-1, 57-2, 102-1, 199-1, and 199-2 contain the entire HBV genome. (C) Purified HBV DNA digested with *Sap*I. The HBV DNA clones are indicated above the lanes.

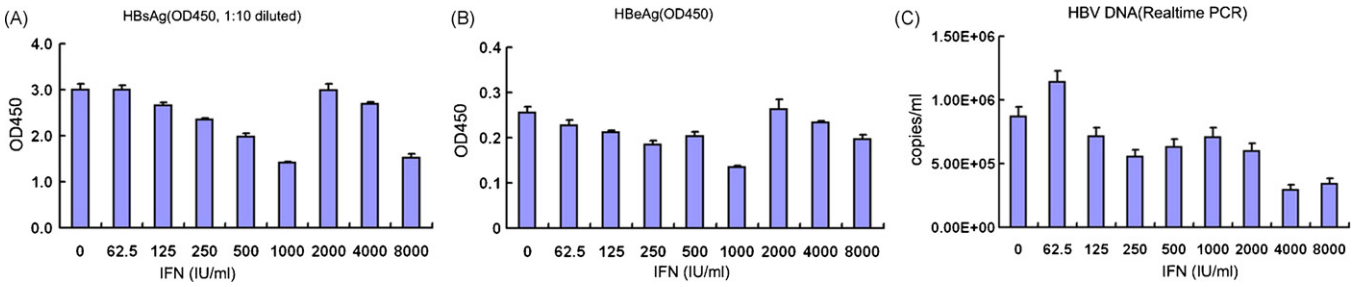

Fig. 3. Inhibitory effect of different concentrations of IFN-alpha on HBV antigen expression and particle secretion. (A) HBsAg secreted into medium. (B) HBeAg secreted into medium. (C) HBV DNA in HBV particles secreted into medium. Means and standard deviations are based on three independent experiments.

was collected for detection of HBsAg, HBeAg and HBV DNA. The concentration of IFN-alpha that was most inhibitory towards HBV was selected by comparing the amounts of HBsAg, HBeAg and HBV DNA secreted into the medium.

2.9. Statistics

Results were reported as means ± standard deviations. The *t*-test was applied to comparisons between groups; a P value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL).

3. Results

3.1. Cloning of wild-type HBV and HBV with BCP 1762/1764 double mutation

Full length HBV genomes of 3.2 kb were amplified from different carriers which named 42, 57, 102, 199 for each using Expand High-Fidelity PCR System (Fig. 2A). After A/T cloning, we acquired constructs harboring HBV full length genome and named as 42-1, 42-2, 57-1, 57-2, 102-1, 199-1, 199-2 for each construct. Insertion of the HBV genome was confirmed by digestion with *Eco*RI (Fig. 2B). The HBV genome from different clone was released and transfected into Huh7 cells. The most competent clone, 57-1, which produced the largest amount of viral antigen and exhibited the highest level of replication was selected and named pGEM-T-HBV (data not shown). PCR-mediated site-directed mutagenesis and molecular cloning using pGEM-T-HBV produced pGEM-T-HBV-TA (clone 4-20) with BCP 1762T/1764A mutations (Fig. 2C). Both of the HBV genome sequence were confirmed by sequencing.

3.2. Replication of wild-type HBV and HBV with BCP 1762/1764 double mutation and the susceptibility to IFN-alpha treatment

3.2.1. Determination of the optimal IFN-alpha concentration in vitro

The inhibitory effect of IFN-alpha on HBV replication and antigen secretion was dose-dependent. As shown in Fig. 3 the inhibitory effect on HBsAg and HBeAg increased as the IFN-alpha concentration increased. The inhibitory effect peaked at 1000 IU ml^{-1}. A similar inhibitory effect was seen for HBV

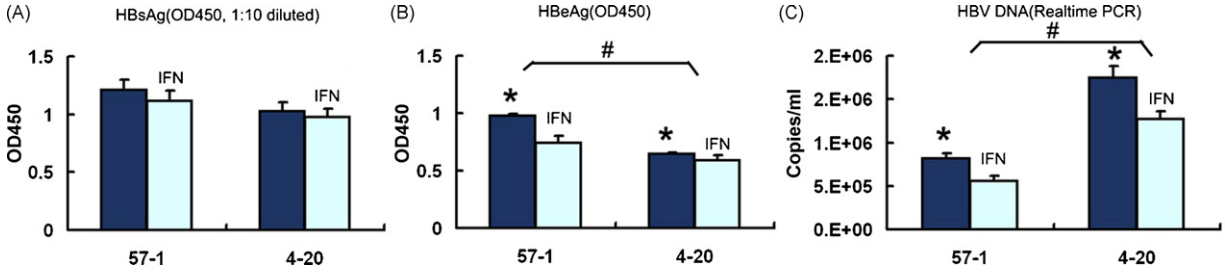

Fig. 4. HBsAg, HBeAg and HBV DNA in the supernatant of transfected cell cultures. (A) HBsAg secreted into medium. (B) HBeAg secreted into medium. (C) HBV DNA in HBV particles secreted into medium. Means and standard deviations are based on three independent experiments. $^{*}P<0.05$ compared to IFN-alpha treatment; $^{\#}P<0.05$ compared to BCP mutant 4-20.

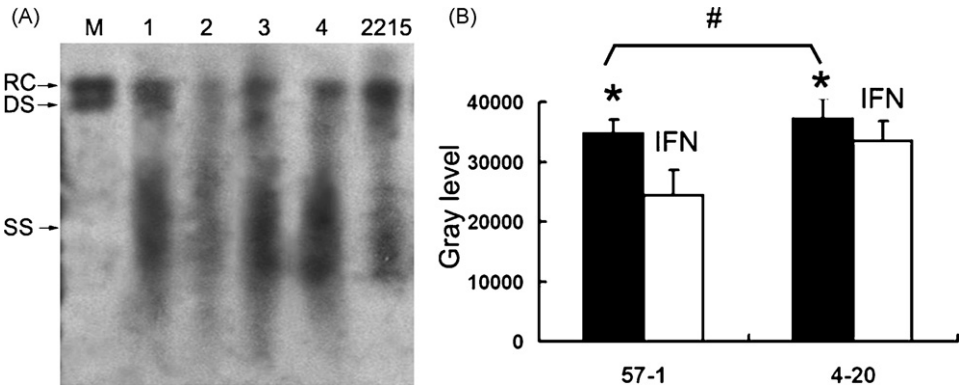

Fig. 5. Southern blot of HBV replicative intermediate. (A) Southern blot of HBV replicative intermediate. M, DNA marker, mixture of 3.2 kb linear HBV DNA and 3.2 kb circular HBV DNA; Lane 1, wild-type HBV 57-1; Lane 2, 57-1 with IFN-alpha treatment; Lane 3, BCP mutant 4-20; Lane 4, 4-20 with IFN-alpha treatment; 2215, HepG2.2.15 (positive control). (B) Relative amount of corresponding HBV replicative intermediate. Means and standard deviations are based on three independent experiments. $^{*}P < 0.05$ compared to IFN-alpha treatment; $^{\#}P < 0.05$ compared to BCP mutant 4-20.

particles secreted into medium; however, the highest inhibitory effect here was at 4000 IU ml^{-1}. Based on these results and those of other researchers, 1000 IU ml^{-1} was selected as the working concentration of IFN-alpha.

3.2.2. HBV antigen secretion and replication of wild-type HBV and the BCP double mutant

The amounts of HBsAg secreted into medium were similar for both wild-type HBV and the BCP double mutant. In contrast, the amount of HBeAg secreted into the medium clearly differed between wild-type HBV and BCP double mutant ($P < 0.05$) (Fig. 4). The BCP double mutant secreted 35.4% less HBeAg than wild-type HBV. The BCP mutation did not affect the expression and secretion of HBsAg. HBV DNA in HBV particles secreted into medium of the BCP double mutant was increased by 113.9% in comparison with wild-type HBV (Fig. 4).

Southern blot analysis was carried out to detect HBV replicative intermediates within HBV core particles in the cytoplasm of transfected Huh7 cells (Fig. 5). Replicative intermediates of the BCP double mutant were increased in comparison with wild-type HBV ($P < 0.05$). These results indicate that the BCP double mutant had a higher replication rate than wild-type HBV.

3.2.3. Inhibitory effect of IFN-alpha on replication of wild-type HBV and HBV with BCP 1762/1764 double mutation

The inhibitory effect of IFN-alpha on viral antigens HBsAg and HBeAg was different in wild-type HBV and BCP double mutant (Fig. 4). The inhibitory effect of IFN-alpha on HBsAg was similar for both wild-type HBV and BCP double mutant. The inhibition of HBeAg was significantly different between wild-type HBV and the BCP double mutant ($P < 0.05$). Wild-type HBV HBeAg was inhibited by 25.7%, while HBeAg of the BCP double mutant was inhibited by only 8.4% (Fig. 4).

HBV DNA in HBV particles secreted into medium was inhibited by IFN-alpha in both wild-type HBV and the BCP double mutant (Fig. 4) ($P < 0.05$). These results indicate that IFN-alpha can inhibit particle secretion of both wild-type HBV and the BCP mutant type. The inhibition of wild-type HBV DNA was somewhat stronger than that of the BCP double mutant (31.8% versus 27.4%).

Southern blot analysis showed that IFN-alpha reduced the amount of HBV replicative intermediates within HBV core particles in the cytoplasm of transfected Huh7 for both wild-type HBV and the BCP double mutant ($P < 0.05$) (Fig. 5). The inhibitory effect on wild-type HBV was stronger (29.8%) than for the BCP double mutant (10.0%). These results indicate that IFN-alpha can inhibit replication of both the wild-type HBV and the BCP mutant.

4. Discussion

This study evaluated the biological properties of HBV variants with mutations in the core promoter since such mutations are commonly observed during chronic HBV infection. The upstream regulatory sequences of the HBV precore and core region include the BCP (1742–1849), core upstream regulatory sequences (CURS, 1643–1742) and a negative regulatory element (NRE, 1611–1634). These sequences are important in viral replication and gene expression and can regulate the transcription of precore mRNA and core mRNA (Yuh et al., 1992). Further studies (Yu and Mertz, 1996) have indicated that unique promoter controlled transcription of precore and pregenome RNA, which overlap in basic elements but are separate genetically, is regulated by different transcription factors. Therefore, mutations of BCP would have different influences on transcription of precore and pregenome RNA. Precore mRNA would be translated and processed into HBeAg while pregenome RNA would serve as the template of reverse transcription for viral replication. Hence, BCP mutations would have different effects on HBeAg and viral replication.

Our research showed that a BCP double mutation (A1762T and G1764A) has no obvious effect on HBsAg production and secretion, but does influence HBeAg production and secretion, and viral replication. Compared to wild-type HBV, secretion of HBeAg into culture medium decreased 35.4% after transfection with HBV DNA harboring the BCP double mutation. There was

also a two-fold increase in viral DNA in the medium and a concomitant rise in viral replicative intermediates in the cytoplasm. Thus, HBV haboring the BCP double mutation synthesizes and secretes less HBeAg and has a higher replication efficiency.

These observations are consistent with previously published data. *In vitro* studies have shown that the A1762T-G1764A double mutation specifically suppresses precore RNA transcription and HBeAg synthesis, and enhances viral replication (Yuh et al., 1992; Buckwold et al., 1996; Baumert et al., 1998). Buckwold et al. (1997) found that the BCP double mutation decreases HBeAg production but does not affect the production of HBcAg and pregenome RNA. Instead, the efficiency of viral replication increased. Furthermore, probably because HBeAg might inhibit viral replication (Guidotti et al., 1996), HBV with decreased HBeAg production would have a higher replication efficiency. But there are contradictory results as well. Nishizono et al. (1995) did not find any significant difference in promoter activities between wild-type HBV and the BCP double mutant. Lindh et al. (1999) found that the A1762T mutation may be useful as a marker for progressive liver damage but not be associated with HBeAg status or virus load. Most of the available information is from clinical studies, and the results stemming from the interaction between the virus and the immune system may not directly reflect the extent of viral replication. Moreover, mixed infections of wild-type and mutant HBV were present, in which the proportion of each would affect the HBV titer in the serum.

The mechanism of the above results is complicated and might involve many factors, especially transcription factors. Moriyama (1997) considered that the increase in viral replication efficiency was due to increased pregenome mRNA and increased HBcAg production. *In vivo* functional studies have shown that T/A double mutations convert the nuclear receptor binding site and change X protein sequences, resulting in the suppression of C gene expression and the restoration of enhanced viral replication (Gunther et al., 1998). Further studies (Li et al., 1999) indicated that this double mutation not only removes the nuclear receptor binding site but also creates an HNF1 transcription factor binding site.

IFNs have immunomodulatory properties as well as direct antiviral activity. Interferons exert their cellular activities by binding to specific membrane receptors on the cell surface. Once bound to the cell membrane, IFNs initiate a complex sequence of intracellular events. *In vitro* studies have demonstrated that these events include the induction of certain enzymes, suppression of cell proliferation, immunomodulation activities such as enhancement of the phagocytic activity of macrophages and augmentation of the specific cytotoxicity of lymphocytes for target cells, and inhibition of virus replication in virus-infected cells. However, there were no conclusions about the BCP mutation and the IFN sensitivity. It is well known that cells expressing only HBcAg do not elicit a response from specific CD4+ T cells, whereas CD4+ T cells recognize and lyse cells harboring HBeAg (e.g. hepatocytes infected with wt virus) (Diepolder et al., 1999). Therefore, lower SR in HBeAg-negative patients is partially due to lower or absent HBeAg expression.

Various *in vitro* studies have examined the inhibitory effects of IFN-alpha/beta on HBV-replication. Rang et al. (1999) have shown that IFN-alpha may reduce the stability of HBV RNA in transiently transfected Huh7 cells, and Caselmann et al. (1992) observed a decrease of pregenomic HBV-RNA in Hep G2 cells stably transfected with the HBV genome when they were treated with IFN-alpha. Hamasaki et al. (1992) demonstrated a reduction of HBs antigen mRNA levels by IFN-alpha in hepatoma cells with integrated HBV DNA. Thus, type I IFNs inhibit HBV replication and protein production by a variety of different mechanisms. Erhardt et al. (2000) suggested that HBV genome mutations located within the BCP were determinants of a response to IFN therapy. Moreover, the BCP mutation might determine different outcomes of IFN therapy in CHB patient with different HBeAg status. They performed a sequence analysis of the NRE, CURE, BCP and precore region from the sera of 96 patients with chronic replicative hepatitis B (64 HBeAg-positive patients and 32 HBeAg-negative patients) treated with IFN-alpha. IFN responsiveness correlated to HBV DNA levels, HBsAg levels, the number of mutations in the complete BCP, especially in nucleotide region 1753–1766, and mutations at nucleotides 1762 and 1764. In HBeAg-positive hepatitis, SR to IFN was associated with a high number of mutations in the BCP ($P < 0.04$) and nucleotide region 1753–1766 ($P < 0.015$), as well as mutations at nucleotide 1764 ($P < 0.007$). In HBeAg-negative hepatitis, SR to IFN correlated with a low number of mutations in the BCP ($P < 0.04$) and nucleotide region 1753–1766 ($P < 0.02$) and with a wild-type sequence at nucleotide 1764 ($P < 0.003$). Prediction of IFN response was possible on the basis of nucleotide 1764 in 77% of HBeAg-positive patients and 78% of HBeAg-negative patients. On the one hand, BCP mutation downregulates precore mRNA transcription and decreased HBeAg production, which could serve to lower the immune tolerance induced by HBeAg and increase the IFN treatment effect. On the other hand, BCP mutation enhances pregenome mRNA transcription and increases viral load, which could decrease the effect of IFN treatment.

Contradictory results include those of Hannoun et al. (2002), which indicate that HBV X gene and BCP mutations are rare in CHB patients and have no significant relation to IFN therapy. Chun et al. (2000) also showed that BCP mutation has no significant relation to viral replication and liver injury.

Besides HBV BCP double mutation, there may exist other mutations affecting the HBV replication and IFN sensitivity. To compare IFN-alpha sensitivity between BCP mutation and HBV wild type, we amplified wild-type HBV DNA with high replicative potency from a chronic HBV infection and acquired the BCP mutation strain by site-directed mutagenesis method from the isolate. Therefore, two sequences are identical except for the BCP. Further confirmation will be necessary with multiple replicates from different patients, including patients with high and low viral load.

In conclusion, current observation has shown that wild-type HBV was more sensitive to IFN-alpha *in vitro* than HBV carrying a BCP double mutation. These findings demonstrate that the BCP mutation may be associated with sensitivity to IFN-alpha treatment, but there is a need for further studies to clarify the particular mechanism(s) involved.

Acknowledgements

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References

Baumert, T.F., Marrone, A., Vergalla, J., Liang, T.J., 1998. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. J. Virol. 72, 6785–6795.

Berger, J., Hauber, J., Hauber, R., Geiger, R., Cullen, B.R., 1988. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene 66, 1–10.

Buckwold, V.E., Xu, Z., Chen, M., Yen, T.S., Ou, J.H., 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J. Virol. 70, 5845–5851.

Buckwold, V.E., Xu, Z., Yen, T.S.B., Ou, J.-H., 1997. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. J. Gen. Virol. 78, 2055–2065.

Caselmann, W.H., Meyer, M., Scholz, S., Hofschneider, P.H., Koshy, R., 1992. Type I interferons inhibit hepatitis B virus replication and induce hepatocellular gene expression in cultured liver cells. J. Infect. Dis. 166, 966–971.

Chun, Y.K., Kim, J.Y., Woo, H.J., Oh, S.M., Kang, I., Ha, J., Kim, S.S., 2000. No significant correlation exists between core promoter mutations, viral replication, and liver damage in chronic hepatitis B infection. Hepatology 32, 1154–1162.

Cullen, B.R., Malim, M.H., 1992. Secreted placental alkaline phosphatase as a eukaryotic reporter gene. Methods Enzymol. 216, 362–368.

Diepolder, H.M., Ries, G., Jung, M.C., Schlicht, H.J., Gerlach, J.T., Graner, N., Caselmann, W.H., Pape, G.R., 1999. Differential antigen-processing pathways of the hepatitis B virus e and core proteins. Gastroenterology 116, 650–657.

Erhardt, A., Reineke, U., Blondin, D., Gerlich, W.H., Adams, O., Heintges, T., Niederau, C., Haussinger, D., 2000. Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. Hepatology 31, 716–725.

Guidotti, L.G., Matzke, B., Pasquinelli, C., Shoenberger, J.M., Rogler, C.E., Chisari, F.V., 1996. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. J. Virol. 70, 7056–7061.

Gunther, S., Li, B.C., Miska, S., Kruger, D.H., Meisel, H., Will, H., 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. J. Virol. 69, 5437–5444.

Gunther, S., Piwon, N., Will, H., 1998. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653) → T, A(1762) → T and G(1764) → A mutations in the core promoter. J. Gen. Virol. 79, 375–380.

Hadziyannis, S.J., Tassopoulos, N.C., Heathcote, E.J., Chang, T.T., Kitis, G., Rizzetto, M., Marcellin, P., Lim, S.G., Goodman, Z., Wulfsohn, M.S., Xiong, S., Fry, J., Brosgart, C.L., Adefovir Dipivoxil 438 Study Group, 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. N. Engl. J. Med. 348, 800–807.

Hamasaki, K., Nakata, K., Nakao, K., Misuoka, S., Tsutsumi, T., Kato, Y., Shima, M., Ishii, N., Tamaoki, T., Nagataki, S., 1992. Interaction of interferon-α with interleukin-1β or tumor necrosis factor-α on hepatitis B virus enhancer activity. Biochem. Biophys. Res. Commun. 183, 904–909.

Hannoun, C., Horal, P., Krogsgaard, K., Lindh, M., 2002. Mutations in the X region and core promoter are rare and have little impact on response to interferon therapy for chronic hepatitis B. J. Med. Virol. 66, 171–178.

Kurosaki, M., Enomoto, N., Asahina, Y., Sakuma, I., Ikeda, T., Tozuka, S., Izumi, N., Marumo, F., Sato, C., 1996. Mutations in the core promoter region of hepatitis B virus in patients with chronic hepatitis B. J. Med. Virol. 49, 115–123.

Li, J., Buckwold, V.E., Hon, M.W., Ou, J.H., 1999. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. J. Virol. 73, 1239–1244.

Lindh, M., Hannoun, C., Dhillon, A.P., Norkrans, G., Horal, P., 1999. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J. Infect. Dis. 179, 775–782.

Marcellin, P., Chang, T.T., Lim, S.G., Tong, M.J., Sievert, W., Shiffman, M.L., Jeffers, L., Goodman, Z., Wulfsohn, M.S., Xiong, S., Fry, J., Brosgart, C.L., Adefovir Dipivoxil 437 Study Group, 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N. Engl. J. Med. 348, 808–816.

Moriyama, K., 1997. Reduced antigen production by hepatitis B virus harbouring nucleotide deletions in the overlapping X gene and precore–core promoter. J. Gen. Virol. 78, 1479–1486.

Nishizono, A., Hiraga, M., Kohno, K., Sonoda, Y.T., Terao, H., Fujioka, T., Nasu, M., Mifune, K., 1995. Mutations in the core promoter/enhancer II regions of naturally occurring hepatitis B virus variants and analysis of the effects on transcription activities. Intervirology 38, 290–294.

Okamoto, H., Tsuda, F., Akahane, Y., Sugai, Y., Yoshiba, M., Moriyama, K., Tanaka, T., Miyakawa, Y., Mayumi, M., 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. 68, 8102–8110.

Rang, A., Gunther, S., Will, H., 1999. Effect of interferon alpha on hepatitis B virus replication and gene expression in transiently transfected human hepatoma cells. J. Hepatol. 31, 791–799.

Summers, J., Mason, W.S., 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29, 403–415.

Wen, Y.M., He, L.F., Lou, H.Z., 1986. The expression of HBV DNA in HepG2 cells. Natl. Med. J. China 69, 260–263.

Will, H., Reiser, W., Weimer, T., Pfaff, E., Buscher, M., Sprengel, R., Cattaneo, R., Schaller, H., 1987. Replication strategy of human hepatitis B virus. J. Virol. 61, 904–911.

Yu, X.M., Mertz, J.E., 1996. Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. J. Virol. 70, 8719–8726.

Yuh, C.H., Chang, Y.L., Ting, L.P., 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J. Virol. 66, 4073–4084.