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Effects of mutations in the X gene of hepatitis B virus on the virus replication

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Summary. – Previously, we have found a new mutation at nt 1726–1730 that is associated with lower hepatitis B virus (HBV) DNA levels in the liver, and mutations at nt 1762/1764 that are correlated with higher HBV DNA levels. To confirm the effects of these mutations on the virus replication efficiency, substitutions nt 1726–1730 CTGAG and A1762T/G1764A in the HBV X (HBX) gene region were investigated alone or in combination. Cells Huh-7 or HepG2 were transfected with these constructs. The effects of these mutations on HBV were investigated at the gene and protein levels. The double mutation A1762T/G1764A increased whereas the nt 1726–1730 CTGAG mutations decreased the levels of released virion-associated and intracellular HBV DNA. The combined mutations had no appreciable effect on the replication capacity of the virus. Cells bearing the constructs with double mutations A1762T/G1764A contained the lowest levels of hepatitis B e antigen (HBeAg). Lowest expression of HBV X protein was in constructs that had both A1762T/G1764A and 1726–1730 CTGAG mutations. We think that changes in secondary RNA structure that were caused by these mutations might have been responsible for those results.

Keywords: hepatitis B virus; X gene; mutants; replication

Introduction

HBV infection is a major global health problem. It causes a wide spectrum of disease manifestations, ranging from asymptomatic infection to acute self-limiting or fulminant hepatitis, or chronic infection with variable disease activity. About 2 billion people have been infected with HBV, and 350 million among them are suffering from chronic HBV infection (Lavanchy *et al*., 2004). HBV has a high mutational rate under pressure of host immunity, due to a high viral copy number produced during active replication, and the lack of proofreading activity in the HBV polymerase (Hannoun *et al.,* 2000; Sheldon *et al.*, 2006). These HBV mutants have epitope alterations that are important for host immune recognition, which enhance virulence with increased levels of HBV replication, resistance to antiviral therapies, or facilitation of cell attachment/penetration. Several lines of evidence support the association between sustained high levels of HBV replication and accompanying hepatitis and risk of cirrhosis and its complications. Ongoing HBV replication may accelerate the progression of chronic hepatitis B to hepatocellular carcinoma (HCC) (Sumi and Yokosuka, 2003).

HBV is a small DNA virus with a 3.2-kb, partially double-stranded, circular genome. This genome contains four overlapping open reading frames that code the surface antigen (pre-S1, pre-S2 and S proteins), core antigens (pre-C and C proteins), reverse transcriptase (P protein), and transactivator (X protein).

One of the most critical mutations of HBV DNA is the double mutation at A1762T and G1764A in the basal core promoter (BCP) region, which affects codons 130 and 131 of the X gene (K130M and V131I). The mutations were first reported by Okamoto *et al.* (1994), who suggested that they might arrest the transcription of the HBV pre-core (pre-C)

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Abbreviations: HBeAg = hepatitis B e antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; BCP = basal core promoter; pre-C mRNA = pre-core mRNA; pgRNA = pregenomic RNA; SEAP = secreted alkaline phosphatase; HPRE = HBV post-transcriptional regulatory element; HBX = hepatitis B virus X gene; wt = wild type; HBV X protein = hepatitis B virus X protein; HCC = hepatocellular carcinoma

mRNA but not seriously affect that of the pregenomic (pg) RNA. Subsequently, studies involving transfection of human hepatoma cell lines and analysis of clinical samples have shown that these double mutations suppress, but do not abolish the synthesis of HBeAg and may also increase HBV DNA replication (Buckwold *et al.*, 1996; Pang and Yuen, 2004). However, other reports do not support the conclusion that BCP mutations increase viral replication, in fact, the BCP double mutations seem to have no effect on viral load (Scaglioni and Melegari, 1994; Yoo and Park, 2003), or even may be associated with lower serum HBV DNA concentrations (Chen and Oon, 2003). Furthermore, others have found in transfection studies that core promoter mutations other than those at nt 1762/1764 seem to upregulate viral DNA replication (Parekh and Zoulim, 2003; Jammeh and Tavner, 2008). Therefore, the effect of the BCP double mutations on viral loads remains uncertain.

In a previous *in vivo* study, we found a new mutation at nt 1726–1730 that was associated with expression of tissue HBV DNA levels, which had not been documented before. The mutation had an opposite effect to the nt 1762/1764 mutation regarding the expression of HBV DNA levels (Rong *et al.*, 2008). We found that the CTGAG mutation at nt 1726–1730 was the most common. The mutation at nt 1726–1730 is in the core upstream regulatory sequence and enhancer II, which may directly affect the regulation of viral replication. In addition, the mutation at nt 1726–1730 results in double amino acid changes in the HBV X protein: aa 118/119 located within the important T*H*-cell epitope aa 111–135 and T*C*-cell epitope aa 115–123. The mutation may affect the ability of the virus to evade immune clearance. All of these are possible mechanisms of the association between nt 1726–1730 and HBV replication.

In this study, we further investigated the effects of the nt 1726–1730 and A1762T/G1764A mutations in the HBX on the virus replication *in vitro*.

Materials and Methods

Plasmid constructs. A replication-competent plasmid (P3.8II, kindly provided by Professor Yuan Wang, Academic Sinica, Shanghai, China; Fu and Cheng, 1998) containing a 1.2× genome length of HBV subtype *adr* (genotype C) in a pBluescript II KS (+) (Stratagene, USA) was used as template for the generation of all mutated constructs. The 3.8 kb HBV insert encompassed nt 1400–3213 and 1–1988 of the HBV genome, starting at the 5′-end from enhancer II upstream of the BCP, and bearing a 3′-terminal redundant region containing the polyadenylation signal for all viral mRNAs. This plasmid was used as template for QuickChange multi site-directed mutagenesis kit from Stratagene, as described in the manufacturer´s instructions. For combined mutations, mutagenesis of the double BCP mutation (A1762T/G1764A) was performed first and the resulting constructs were used as templates for introduction of nt 1726–1730 CTGAC mutations. The primers for site-directed mutations were: Muta1 and Muta1′ for nt 1726–1730 CTGAG mutations; Muta2 and Muta2′ for A1762T/G1764A mutations. All constructs were sequenced to ensure that no additional mutations were introduced in the process.

Cell cultures and transfection. The Huh7 and the HepG2 hepatoma cell lines were grown and maintained in DMEM containing 10% FBS, 2 mmol/l L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells grown to approximately 90% confluence in six-well plates were transfected.

Secreted alkaline phosphatase (SEAP) assay. A plasmid-liposome complex [3 μg recombinant plasmid and 1 μg of SEAP complexed with 7.5 μg of Lipofectin (Invitrogen, USA)] was prepared in serum-free OptiMEM medium (Invitrogen). Medium (2 ml/well) was replaced 6 hrs later, and the cells were harvested after 48 hrs. Cell pellets were used for the SEAP assay as well as for the extraction of HBV DNA. The experiments were repeated three times.

Assay of virion-associated HBV DNA. Aliquots of 750 μl of medium from transfected cells were digested with 1.5 U DNaseI (Ambion,USA) for 30 min at 37°C. After digestion, DNaseI was heat-inactivated at 75°C for 5 min. HBV DNA was extracted using a MiniBEST viral RNA/DNA extraction kit according to the manufacturer's instructions (Takara, Japan). Purified HBV DNA was resuspended in 20 μl of ultrapure water and stored at -20°C.

Intracellular HBV DNA assay. The cells were harvested by 0.25% trypsin and resuspended in 200 μl of elution buffer. The lysate was digested with DNaseI (1.5 U) at 37°C for 15 min to degrade transfected HBV DNA. After addition of 600 μl of lysis buffer [10 mmol/l Tris/HCl (pH 8.4), 1.5 mmol/l $MgCl_2$, 0.14 mol/l NaCl, and 1% NP-40] and 6 μl of proteinase K (20 mg/ml), samples were digested at 55°C for 3 hrs. Proteinase K was heat-inactivated at 95°C for 10 min. The samples were treated with RNase at 37°C for 1 hr to digest the RNA. The DNA was extracted with phenol and precipitated with ethanol.

The relative levels of extracellular and intracellular HBV DNA were analyzed by fluorescence real-time quantitative PCR with a QuantiTech SYBR green PCR master-mix kit (Takara). The primers for HBV DNA were HBs1 and HBs2. The PCR contained 0.5 μl of each primer at 0.5 mmol/l, 2 μl of extracted HBV DNA in a 20-μl reaction. Cycling consisted of a holding step at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. This was followed by a data acquisition step at 80°C for 15 sec to check the specificity of the PCR product.

Real-time RT-PCR. HBV transcript RNA was prepared by extraction of total cellular RNA using a ReasyMicro kit (Qiagen, Germany) according to the manufacturer´s instructions. Total HBV RNA (3 μg) and primer oligo(dT) were made up to 12 μl with RNase-free water and incubated for 5 min at 70°C. The mixture was then ice-cooled and the volume was made up to 25 μl by adding 5.5 μl of RNase-free water, 1.5 μl of dNTP, 1 μl of reverse transcriptase and

Table 1 Primers used in this study

Primers		
Muta1	5′-CTTCAAAGACTGTTTGTTTACTGAGTGGGAGGAGTTGGGG-3′	for nt 1726–1730 CTGAG mutations
Muta1′	5′-CCCCAACTCCTCCCAGTCAGTAAACAAACAAACAGTCTTTGAAG-3′	
Muta2	5′-TGGGGGAGGAGATTAGGTTAATGATCTTTGTACTAGGAGG-3′	for A1762T/G1764A mutations
Muta2′	5′-CCTCCTAGTACAAAGATCATTAACCTAATCTCCTCCCCCA-3′	
HBs1	5′-CTTCATCCTGCTGCTATGCCT-3′ (nt 406–426)	for HBV DNA
HBs2	5′-AAAGCCCAGGATGATGGGAT-3′ (nt 608–627)	
PCP	5′-GTCTGTTCACCAGCACCA-3′ (nt 1797–1814)	for pre-C mRNA specifically
BC1	5′-GGAAAGAAGTCAGAAGGCAA-3′ (nt 1954–1974)	
PGP	5′-CACCTCTGCCTAATCATCTCA-3′ (nt 1826–1846)	for both pre-C mRNA and pgRNA
HBX1	5′-GCACTTCGCTTCACCTCT-3′ (nt 1583–1600)	for HBX mRNA
HBX2	5′-TATGCCTACAGCCTCCTA-3′ (nt 1793–1776)	

5 μl of 5× RT buffer (Promega, USA). The mixture was incubated at 37°C for 1 hr in a thermocycler for cDNA synthesis.

For the detection of HBV pgRNA, HBV pre-C mRNA and HBX mRNA, 1 μl of cDNA was used as a template in LightCycler reactions set up as described above. The primers used were PCP and BC1, as described by Carman and Jacyna (1989) for HBV pre-C mRNA specifically, and PGP with BC1 for HBV pre-C mRNA, and for HBV pgRNA, primers described by Laras and Koskinas (2002). The primers for HBX mRNA were HBX1 and HBX2, as described by Rong *et al.* (2008).

Control of contamination of HBV DNA is especially important for HBV mRNA expression because it does not consist of introns. Controls were performed by omitting reverse transcriptase from the above incubation medium used to synthesize cDNA. The products were quantified by the same fluorescent quantitative real-time PCR assay kits. All the primers used in the study are shown in Table 1.

Western blot analysis. The cells were lysed in RIPA lysis buffer [50 mmol/l Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 0.1% SDS, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 mmol/L Na_3VO_4, and 1 mmol/l NaF]. The proteins were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membranes that were blocked in Tris-buffered saline with Tween-20 containing 5% non-fat dried milk. The blotted membranes were incubated with polyclonal anti-HBx antibody (a kind gift from Dr Mark Feitelson, Thomas Jefferson University, USA; Feitelson and Clayton, 1990).

ELISA. The culture supernatants were tested for hepatitis B surface antigen (HBsAg) using the diagnostic kit for HBsAg (ELISA) from Shanghai Kehua Biotech. Secreted HBeAg was detected by the diagnostic kit for HBeAg (ELISA) from the same manufacturer. The results were expressed as the ratio of sample to standard.

Secondary RNA structure. Computer models of RNA folding were generated using the RNAdraw software (Matzura and Wennborg, 1995), which is based on the Vienna RNA package. All calculations were performed for a temperature of 37°C.

Statistical analysis. The data were expressed as mean ± SD. Differences between any two groups were determined by Wilcoxon′s test; differences with $P < 0.05$ were considered significant.

Results

Transfection efficiency

The transfection efficiency was monitored by reporter plasmids expressing SEAP. The results are shown in Fig. 1. The transfection efficiencies between various constructs were similar.

Virion-associated and intracellular HBV DNA

The replication capacity of each construct, measured in terms of virion-associated HBV DNA released in the culture medium at 48 hrs post transfection is shown in Fig. 2. The construct bearing the CTGAG mutation at nt 1726–1730 yielded fewer virions (5.56 ± 5.29 $\log_{10}$ copies/ml) compared with the wild type (wt) (5.72 ± 5.24 $\log_{10}$ copies/ml) ($P = 0.021$), although the wt only released 1.05-fold more

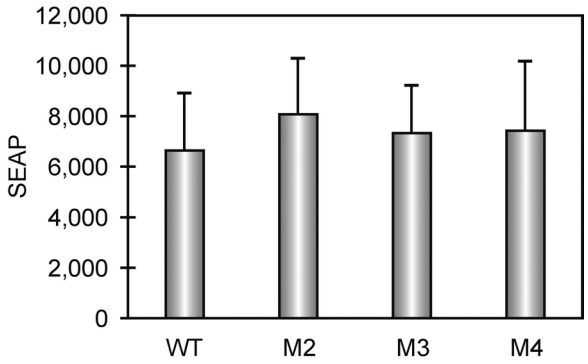

Fig. 1

Expression of SEAP by the plasmid constructs

The plasmids harboring wt HBV (wt), CTGAG mutation at nt 1726–1730 (M2), double mutation A1762T/G1764A (M3) and their combination (M4) were examined.

virions. The construct with double BCP mutation (A1762T/G1764A) (5.81 ± 5.0 $\log_{10}$ copies/ml) released 1.39-fold (P = 0.017) more virions than nt 1726–1730 CTGAG mutations and 1.22-fold more (P = 0.011) than wt. There was no significant difference in virion yield between the wt and the construct bearing the nt 1726–1730 CTGAG mutations and the double mutation (A1762T/G1764A) together (P = 0.206).

Measurement of intracellular HBV DNA levels followed the same trend as that observed for released HBV DNA, without exception. Fig. 3 shows the results obtained with the various constructs containing mutations. The results were expressed as a ratio to GAPDH levels. The range was from 5,600 to 12,000. HBV with nt 1726–1730 CTGAG mutations had the lowest level of HBV DNA expression ($5.60\times10^3 \pm 7.08\times10^2$) (P = 0.028), whereas HBV with A1762T/G1764A double mutations had the highest expression ($1.30\times10^4 \pm 1.48\times10^3$) (P = 0.018). Wt had similar replication capacity to the constructs bearing the nt 1726–1730 CTGAG mutations and the double mutation (A1762T/G1764A) together (P = 0.753).

HBV pgRNA

Figs. 4–6 show the results obtained for HBV pgRNA, HBV pre-C mRNA and HBX mRNA transcript measurements. Whatever the construct, the HBV pre-C mRNA and the HBX mRNA ratios were significantly lower than those for HBV pgRNA. As for HBV pgRNA levels, the results were in full agreement with those obtained for virion released and intracellular HBV DNA measurements. The construct that had yielded more virions into the medium and higher levels of intracellular HBV DNA also had higher levels of HBV pgRNA transcripts (Fig. 4).

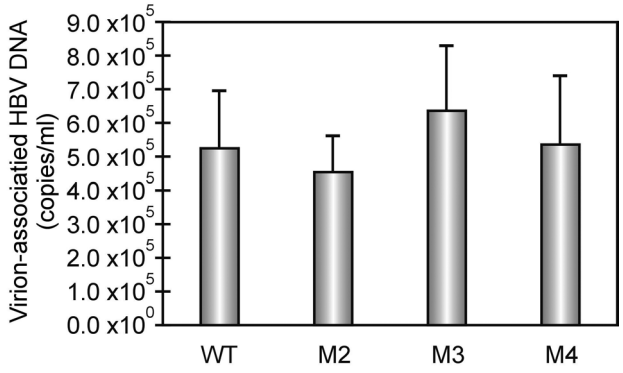

Fig. 2

Effects of the mutations on virion-associated HBV DNA

HBV DNA levels in culture medium were assayed 48 hrs post transfection by real-time PCR. For the legend see Fig. 1.

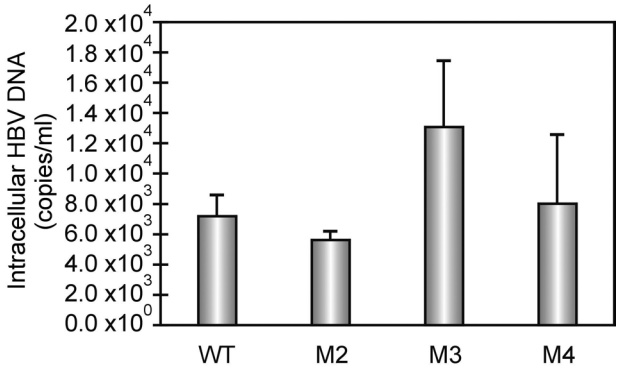

Fig. 3

Effects of the mutations on intracellular HBV DNA

For the legend see Fig. 2.

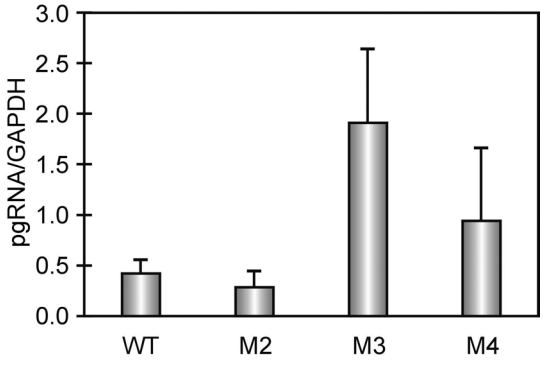

Fig. 4

Effects of the mutations on the expression of HBV pgRNA

HBV pgRNA was assayed at 48 hrs post transfection by RT-PCR. For the legend see Fig. 1.

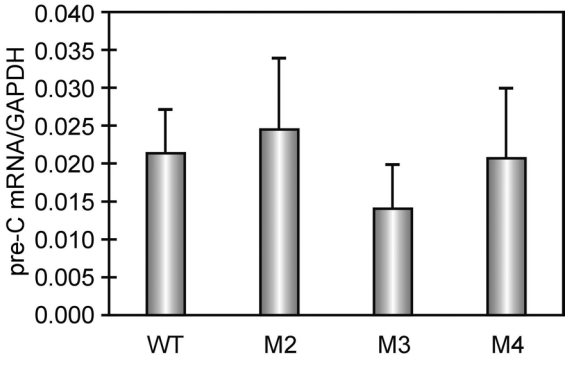

Fig. 5

Effects of the mutations on expression of HBV pre-C mRNA

HBV pre-C mRNA was assayed at 48 hrs post transfection by RT-PCR. For the legend see Fig. 1.

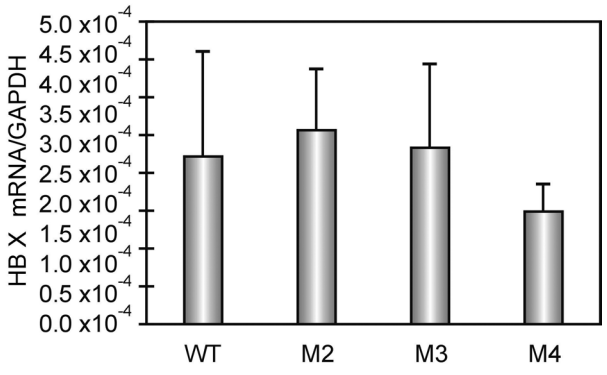

Fig. 6

Effects of the mutations on expression of HBV X mRNA

HBV X mRNA was assayed at 48 hrs post transfection by RT-PCR. For the legend see Fig. 1.

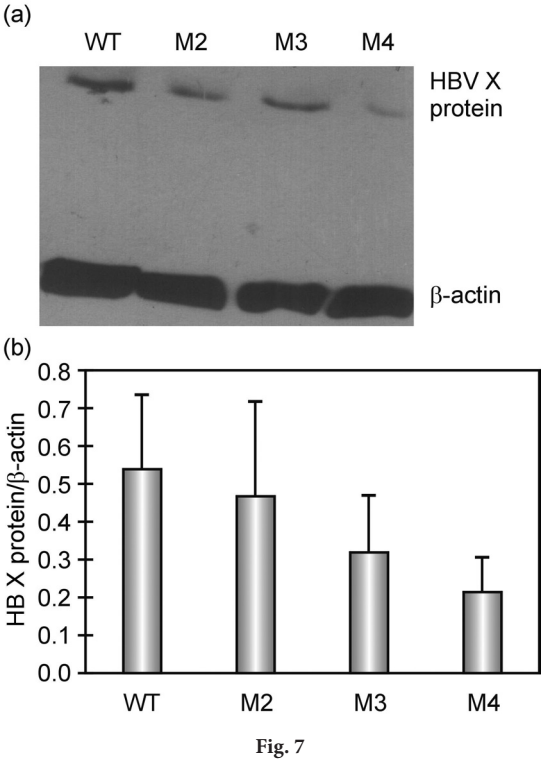

Fig. 7

Effects of the mutations on expression of HBV X protein

HBV X protein was assayed at 48 hrs post transfection by Western blot analysis. For the legend see Fig. 1.

Mutants A1762T/G1764A produced 4.4-fold higher levels (1.91 ± 0.96) of transcript than the wt (0.44 ± 0.15) (P = 0.043). In contrast, the mutation at nt 1726–1730 produced 72% less HBV pgRNA transcript (0.31 ± 0.15) than the wt (P = 0.028). There was no significant difference in the levels detected between the wt and constructs carrying both the nt 1726–1730 CTGAG and A1762T/G1764A mutations (P = 0.333).

HBV pre-C mRNA

HBV pre-c mRNA level (Fig. 5) was reduced in the A1762T/G1764A mutants (0.014 ± 0.005) compared with the wt (0.021 ± 0.005). Thus, 67% lower levels of HBV pre-C mRNA were detected with the nt 1726–1730 CTGAG construct (0.025 ± 0.009, P = 0.016). No significant change was recorded for the other constructs in comparison with the wt.

HBV X mRNA

HBV X mRNA level was the lowest among the three mRNAs (Fig. 6). Higher levels were recorded for the mutation at nt 1726–1730, but the difference was not significant (P >0.05). Similarly, no significant change was found for the other constructs in comparison with the wt.

HBV X protein

Expression of HBV X protein was detected by western blot analysis (Fig. 7). The analysis was carried out using Gelpro4 software. Initially, we thought that HBV X protein expression would be consistent with the trend of HBV X mRNA. Surprisingly, we found that the construct with the combined mutation had the lowest expression of HBV X protein. Compared with the wt, the difference was significant (P = 0.043). There was no significant change for the other constructs in comparison with the wt (P >0.05).

HBsAg and HBeAg

The cell-culture supernatants, 48 hrs post transfection, were tested for HBsAg (Fig. 8). The readings show the index values (A_{450} test reading divided by the standard). The results showed that there was no significant difference in transfection efficiencies between the constructs, which was indicated by the earlier SEAP results.

HBeAg was detected in cell-culture media. The results were expressed in the same manner as for HBsAg. Reduced HBeAg levels were recorded with BCP mutants A1762T/G1764A compared with the wt (P <0.05), reflecting the results obtained for HBV pre-C mRNA measurements. The data are shown in Fig. 9.

Secondary RNA structure

Using RNAdraw, we analyzed the folding of the HBX of our plasmid. The HBX lies between nt 1372 and nt 1834 and contains 462 bases. We predicted the secondary structure of

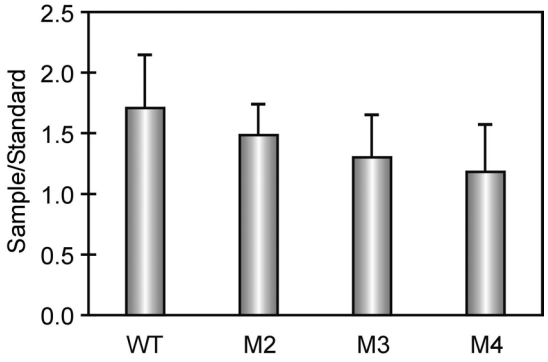

Fig. 8

Effects of the mutations on expression of HBsAg

HBsAg was assayed at 48 hrs post transfection by ELISA. For the legend see Fig. 1.

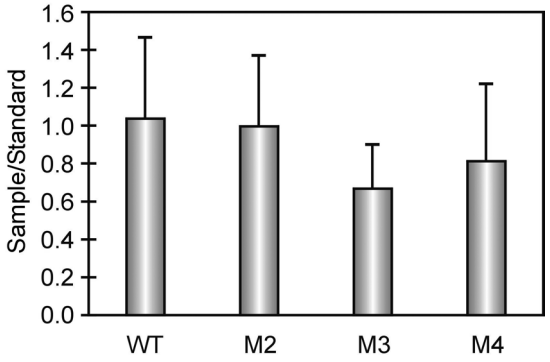

Fig. 9

Effects of the mutations on expression of HBeAg

HBeAg was assayed at 48 hrs post transfection by ELISA. For the legend see Fig. 1.

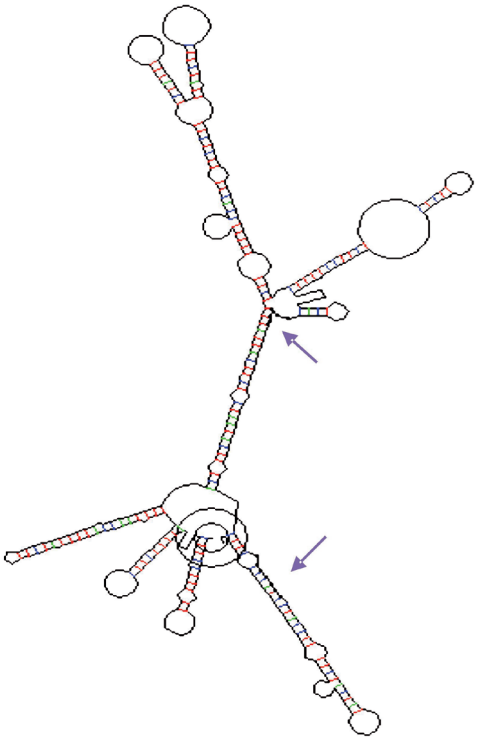

Fig. 10

Secondary structure of X gene RNA of wt HBV

The arrows show nt 1726–1730 and 1762/1764.

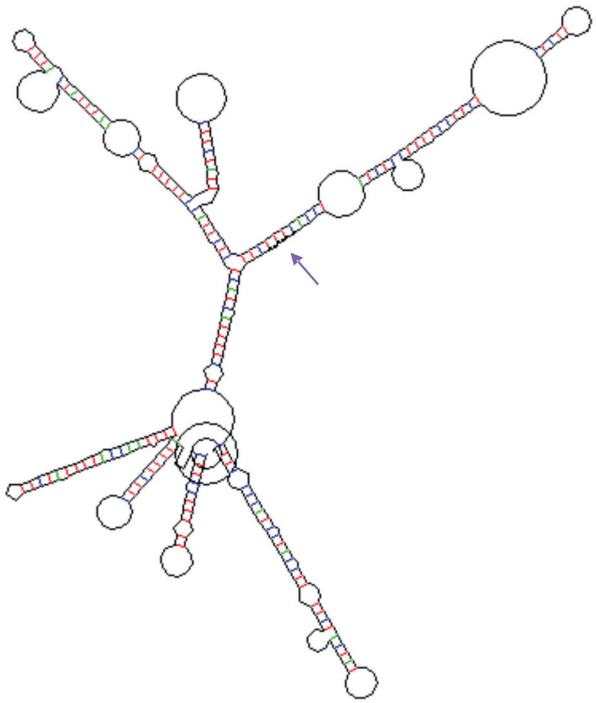

Fig. 11

Secondary structure of X gene RNA of nt 1726–1730 mutant of HBV

The arrow shows CTGAG mutation at nt 1726–1730.

RNA with mutation at nt 1726–1730 and A1762T/G1764A mutations alone and together. Although the secondary structure of HBX RNA has many forms, we still could see the symmetry and conservatism. The four maps that we obtained were symmetrical. Looking at the A1762T/G1764A mutations, compared with wt (AGG), the mutations (UGA) formed a novel stem-loop structure. After further observation, we found that AGG would be part of the base pair forming the stem, whereas UGA took part in the new loop construction. The map containing the mutation at nt 1726–1730 alone was the most striking of the four. The loop in the middle disappeared; all six bases were base pairing,

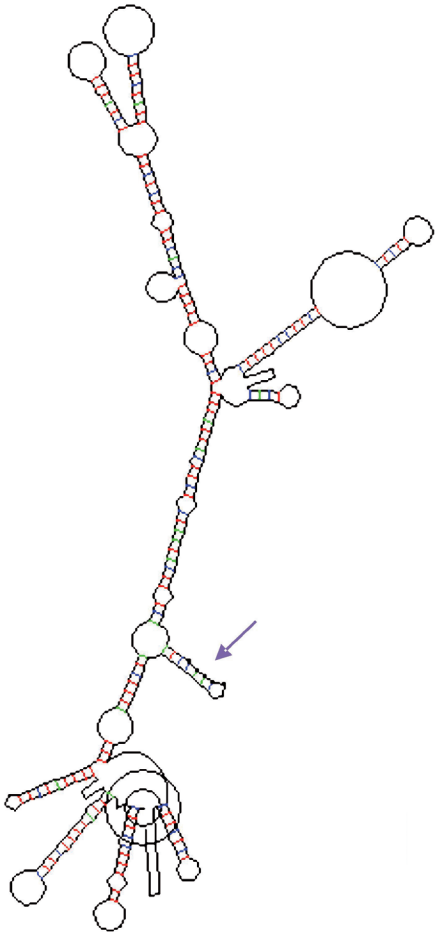

Fig. 12

Secondary structure of X gene RNA of double mutant A1762T/G1764A of HBV

The arrow shows 1762/1764 mutation.

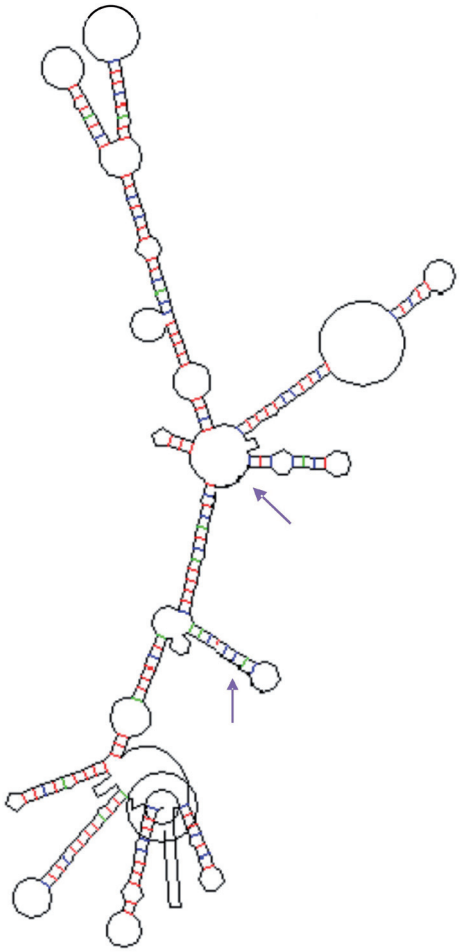

Fig. 13

Secondary structure of X gene RNA of the combined mutant

The arrows show CTGAC mutations at nt 1726–1730 and nt 1762/1764.

taking part into the conformation of stem. Surprisingly, when combined with the A1762T/G1764A mutations, the middle loop reappeared in the secondary structure of the nt 1726–1730 CTGAG mutations. The results are shown in Figs. 10–13.

Discussion

HBV is a prototype member of the family *Hepadnaviridae*. Infection with HBV leads to a wide spectrum of clinical presentations, ranging from an asymptomatic carrier state to acute self-limiting infection or fulminant hepatic failure, chronic hepatitis with progression to cirrhosis, and HCC (Sumi and Yokosuka, 2003; Ganem and Prince, 2004;). It is well known that HBV takes the greatest advantage of the gene structure. It has four overlapping ORF and many kinds of response elements in its 3.2 kb genome. A mutation may have little effect on one viral protein but may have severe consequences on an overlapping gene or on regulatory and structural sequences. Several lines of evidence support that the higher the level of HBV replication, the greater the risk of cirrhosis, HCC, decompenzation, and liver related mortality (Brunetto and Oliveri, 2002; Iloeje and Yang, 2006; Liaw and Tai, 1988; Fattooich and Brollo, 1991).

In a previous *in vivo* study, we found that mutation at nt 1726–1730 had an opposite effect to the nt 1762/1764 mutation regarding the HBV DNA levels, and the occurrence of nt 1726–1730 mutation correlated negatively with that of nt 1762/1764 mutation in all cases (Rong *et al.*, 2008). In the present study, all of the mutations were introduced by site-directed mutagenesis in the same genetic background as an infectious clone, either alone or in combination, to determine their effect on replication. Quantitative measurements of

released virions and intracellular replicative intermediates were undertaken using the fluorescent quantitative real-time PCR following transfection of HBV constructs bearing mutations in the HBX region. In addition, we addressed the issue of the BCP double mutation and its impact on replication; a topic of some controversy.

Several lines of evidence *in vivo* and *in vitro* have indicated that the double mutations at nt 1762/1764 appeared to upregulate viral genome replication and downregulate HBeAg expression (Buckwold *et al.,* 1996; Parekh and Zoulin, 2003), which is similar to our conclusion. We also found that mutation at nt 1726–1730 reduced HBV DNA replication, which was consistent with the result of our previous *in vivo* study. However, it should be emphasized that, although the differences in the two mutations were significant compared with the wt, the fold differences were low. The wt only released 1.05-fold more virions than the mutation at nt 1726–1730 construct, and the construct with the double BCP mutation (A1762T/G1764A) released 1.22-fold more virions than the wt. For intracellular HBV DNA, the level for mutation at nt 1726–1730 construct was 83% lower than for the wt, whereas for A1762T/G1764A mutation, the level of HBV DNA was 1.8-fold higher than for the wt. However, our *in vivo* study showed that the fold difference ranged from 10 to 100 (Rong *et al*., 2008). One of the reasons that our results were much lower than the *in vivo* study, is that the statistical data in the present study were the ratio of GAPDH in cultured cells to the numbers of virions expressed as copies/ml. However, the data used in our previous *in vivo* study were the ratio of HBV DNA copies to the volume of liver tissue. In addition, the pathogenesis of HBV infection is usually caused by the interaction between virus and host immune response. We conclude that mutation at nt 1726–1730 and A1762T/G1764A mutations have a significant effect on HBV DNA replication, but they are not the only factor that causes a major change in replication *in vivo*, which may also be related to the immune response of the host, such as virus evading immune clearance, the interactions between virus and host, and mutations accumulating in the host. We also constructed a plasmid that contained mutations at nt 1726–1730 and A1762T/1764A together. The results showed that there was no significant difference between the combined mutations and the wt, except for the expression of HBV X protein. For the HBV DNA level, the result was consistent with our previous *in vivo* study. HBV genomes with wt nt 1726–1730 accompanied by nt 1762/1764 mutations presented the highest viral load in liver (Rong *et al*., 2008). Interestingly, the HBV X protein of the combined construct was the lowest in all four constructs, and the difference was significant. We measured the level of HBV X mRNA, and found that it was lowest in the combined construct. This might be the result of the decreased HBV X mRNA and the changes in its post-transcription processes and translation. Considering the complicated functions of HBV X protein, we think, that the mutations result in some particular changes in the HBV X protein, and the variation in HBV X protein in turn influences its normal function, such as activating viral or cellular transcription factors and DNA repair. So finally, the HBV X protein affects its own transcription and translation.

To explain the change in viral replication seen with the constructs, we analyzed the secondary RNA structure of the constructs using RNA draw. When A1762T/G1764A was induced, the RNA structure resulting from the computer model indicated a novel stem–loop structure. When UAAAGGU were in the positions 1758–1765, the DR1 sequence (5′-UUCACCUCUGC-3′, 11 nt) formed a half loop and stem structure, and the first U was base paired with 1760A, according to the folding structures. The predicted shift in secondary structure as a result of UGA identity in 1762–1764 transferred these bases from DR1 to a newly created stem-loop side branch. In the second conformation, the DR1 sequence was in dissociation with its 11 nt. The result of the secondary RNA structure analysis was similar to the conclusion of Kidd and Kidd-Ljunggren (1996). It was suggested that the reconfiguration caused by A1762T/G1764A facilitates DR1 binding with its primer and promotes the replication of HBV. We investigated CTGAG mutation at nt 1726–1730. When AGGAC were at positions 1726–1730, the AG took part in the formation of a loop. The latter GC was base paired, and the final A was skipped. When CUGAG mutations occurred in positions 1726–1730, the loop disappeared, and the nucleotides were base paired to form a stem with two skipped nucleotides. It appeared that the change from loop to stem influenced the stability of the RNA structure. It takes more energy to disassemble double strands to single strands during reverse transcription, which decreases virus replication. It has been reported that the HBV post-transcriptional regulatory element (HPRE) might enhance HBV gene expression by facilitating export of intronless viral subgenomic RNAs from the nucleus to the plasma. This genetic element is cis-acting at the post-transcriptional level, and consists of five segments, each of which binds to transcription factors, except for segment II. The function of HPRE depends on the numbers of segments binding to transcription factors (Huang and Yen, 1995). When mutations appeared, the secondary structure of RNA changed and influenced the binding of HPRE to nuclear factors, and resulted in decreased export of mRNA to the plasma. The above process led to downregulation of the course of HBV replication. It was interesting that when CTGAG mutation at nt 1726–1730 occurred simultaneously with A1762T/G1764A mutations, the lost loop reappeared at positions 1726–1730, and the CTGAG mutations were reconfigured into their original structure, but were shifted and paired with other nucleotides. The reconfiguration might lead to HBV DNA levels that are not significantly different from

those of the wt. The structures proposed in this paper can be considered as variants of a working model, which needs to be confirmed. The result of the computer analysis in this paper is convincing evidence that the structures may exist in the real life.

In summary, we found that the CTGAG mutation at nt 1726–1730 correlated with decreased HBV DNA levels, and mutations at A1762T/G1764A correlated with higher HBV DNA levels. This provides new information for the relationship between HBX mutations and HBV replication. It was also found that the mutations at nt 1726–1730 combined with nt A1762T/G1764A mutations reduced expression of HBV X protein. In addition, it appears that such mutations may change the secondary RNA structure that might, in turn, regulate HBV transcription and replication differentially. Mutations related to viral load might lead to new development in diagnosis and therapy of patients with chronic HBV infection. It is accepted that combination of mutations rather than a single mutation is associated with the development of progressive liver disease (Chen and Liu, 2006). Discovery of more mutations or a combination of mutations is essential for the future studies.

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References

Buckwold VE, Xu Z, Chen M (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, 5458–5851.

Brunetto MR, Oliveri F (2002): Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. J. Hepatol. 36, 263–270. http://dx.doi.org/10.1016/S0168-8278(01)00266-5

Carman WF, Jacyna MR (1989): Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 2, 588–591. http://dx.doi.org/10.1016/S0140-6736(89)90713-7

Chen BF, Liu CJ (2006): High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. Gastroenterology 130, 1153–1168. http://dx.doi.org/10.1053/j.gastro.2006.01.011

Chen WN, Oon CJ (2003): Mutations and deletions in core promoter and precore stop codon in relation to viral replication and liver damage in Singaporean hepatitis B virus carriers. Eur. J. Clin. Invest. 30, 787–792. http://dx.doi.org/10.1046/j.1365-2362.2000.00723.x

Fattooich G, Brollo L (1991): Natural history and prognosis factors of chronic hepatitis type B. Gut 32, 294–298. http://dx.doi.org/10.1136/gut.32.3.294

Feitelson MA, Clayton MM (1990): X antigen/antibody markers in hepadanavirus infection: Presence and significance of hepadnavirus X gene product(s) in serum. Gastroenterology 98, 1071–1078. http://dx.doi.org/10.1016/0016-5085(90)90035-Y

Ganem D, Prince AM (2004): Hepatitis B virus infection natural history and clinical consequences. N. Engl. J. Med. 350, 1118–1129. http://dx.doi.org/10.1056/NEJMra031087

Hannoun C, Horal P, Lindh M (2000): Long-term mutation rates in the hepatitis B virus genome. J. Gen. Virol. 81, 75–83.

Huang ZM, Yen TS (1995): Role of the hepatitis B virus posttranscriptional regulatory element in export of intronless transcript. Mol. Cell. Biol. 15, 3864–3869.

Iloeje UH, Yang HI (2006): Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology 130, 678–686. http://dx.doi.org/10.1053/j.gastro.2005.11.016

Jammeh S, Tavner F (2008): Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. J. Gen. Virol. 89, 901–909. http://dx.doi.org/10.1099/vir.0.83468-0

Kidd AH, Kidd-Ljunggren K (1996): A revised secondary structure model for the 3'-end of hepatitis B virus pregenomic RNA. Nucleic Acids Res. 24, 3295–3301. http://dx.doi.org/10.1093/nar/24.17.3295

Laras A, Koskinas J (2002): In vivo suppression of precore mRNA synthesis is associated with mutations in the hepatitis B virus core promoter. Virology 295, 86–96. http://dx.doi.org/10.1006/viro.2001.1352

Lavanchy D (2004): Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J. Viral Hepatol. 11, 97–107. http://dx.doi.org/10.1046/j.1365-2893.2003.00487.x

Fu L, Cheng YC, (1998): Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(-)SddC (3TC) resistence. Biochem. Pharmacol. 55, 1567–1572. http://dx.doi.org/10.1016/S0006-2952(98)00050-1

Liaw YF, Tai DI (1988): The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 8, 493–496. http://dx.doi.org/10.1002/hep.1840080310

Matzura O, Wennborg A (1995): RNADRAW V.1.1. http://mango.mef.ki.se/-ole/madraw/madraw.html.

Okamoto H, Tsuda F, Akahane Y (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.

Pang A, Yuen MF (2004): Real-time quantification of hepatitis B virus core-promoter and pre-core mutants during hepatitis E antigen seroconversion. J. Hepatol. 40, 1008–1017. http://dx.doi.org/10.1016/S0168-8278(04)00098-4

Parekh S, Zoulim F (2003): Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J. Virol. 77, 6601–6612. http://dx.doi.org/10.1128/JVI.77.12.6601-6612.2003

Rong Z, Hui-Ping Z, Hui Y (2008): Hepatitis B virus mutations associated with in situ expression of hepatitis B core

antigen, viral load and prognosis in chronic hepatitis B patients. Pathol. Res. Pract. 204, 731–742. http://dx.doi.org/10.1016/j.prp.2008.05.001

Scaglioni PP, Melegari M (1994): Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. Virology 233, 374–381. http://dx.doi.org/10.1006/viro.1997.8594

Sheldon J, Rodes B, Zoulim F, Bartholomeusz A, Soriano V (2006): Mutations affecting the replication capacity of the hepatitis B virus. J. Viral Hepatol. 13, 427–434. http://dx.doi.org/10.1111/j.1365-2893.2005.00713.x

Sumi H, Yokosuka O (2003): Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 37, 19–26. http://dx.doi.org/10.1053/jhep.2003.50036

Yoo BC, Park JW (2003): Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J. Hepatol. 38, 98–103. http://dx.doi.org/10.1016/S0168-8278(02)00349-5