The impact of mutations near the AUG codon of precore region on hepatitis B e antigen production

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This certifies that the Doctoral Dissertation of

'Ahn, Sang Hoon' is approved.

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I am dedicating this thesis to my parents, younger brother and his wife who fully understood and supported me with patience and generosity.

Ahn, Sang Hoon

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Abstract

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Black South African (SA) carriers of hepatitis B virus (HBV) seroconvert from hepatitis B e antigen (HBeAg) to antibody against HBeAg(anti-HBe) much earlier than do Asian patients. Since majority of South African HBV strains harbor double or triple nucleotide changes immediately upstream of the precore AUG, the translational initiation codon for HBeAg, their impact on HBeAg expression was investigated by site-directed

mutagenesis and transfection experiments in human hepatoma cells. Triple mutations at the -5, -3, and -2 positions of the AUG codon severely impaired HBeAg expression. The frequent double mutation at the -5 and -2 positions also reduced HBeAg levels moderately, to an extent comparable to that of the common core promoter mutations ($1762^{T}/1764^{A}$). Presence of both South African and core promoter mutations diminished HBeAg expression in an additive manner. Although the South African sequence changes caused amino acid substitutions in HBx protein, their effect on HBeAg was independent of HBx expression. Interestingly, the triple South African mutations enabled core protein translation from precore mRNA, suggesting ribosomal leaky scanning of the precore AUG codon. Reduced HBeAg translation may contribute to earlier seroconversion to anti-HBe in this region of the world, and provide the molecular

basis for a previously unexplained clinical event associated with viral clearance.

Key Words: hepatitis B virus, hepatitis B e antigen, South African, precore/core promoter mutation, AUG codon, ribosomal leaky scanning, seroconversion.

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I. Introduction

Hepatitis B virus (HBV) is the prototype member of hepadnaviridae, a group of hepatotropic enveloped DNA viruses. The HBV genome consists of a partially double-stranded 3.2kb DNA molecule arranged in a relaxed circular conformation. It contains four overlapping reading frames for the polymerase (P gene), core protein and HBeAg (pre-C/C gene), large, middle, and small surface proteins (pre-S/S gene), and the X protein (X

gene). These proteins are expressed from 3.5 kb pregenomic RNA (core and polymerase proteins), 3.5 kb precore RNA (Hepatitis B e antigen), 2.4 kb subgenomic RNA (large envelope protein), 2.1 kb subgenomic RNA (middle and major envelope proteins), and 0.7 kb subgenomic RNA (X protein).[1]

HBV infection may lead to a spectrum of liver diseases, ranging from acute self-limited hepatitis, an asymptomatic carrier state to chronic hepatitis progressing to liver cirrhosis and hepatocelluar carcinoma. HBV related liver diseases are the outcome of complex interplay between the virus and its host. The ever-changing host environment selects for outgrowth of different viral variants. During the last decade, with the advent of polymerase chain reaction (PCR) technology, genetic variability of the HBV genome has gained increasing recognition and attention. Major HBV variants have been identified that survive among the host hepatitis B e antibody (anti-HBe) immunity [precore mutants unable to express hepatitis B e Ag and core promoter mutants with reduced HBeAg expression], HBV vaccine (vaccine escape mutants), and Lamivudine

treatment (YMDD motif mutants). However, with a few exceptions, the functional consequences of these prevalent and hence important mutations remain poorly characterized.

Hepatitis B e antigen (HBeAg) was discovered three decades ago[2] and corresponds to an alternative translational product of the core gene (Figure 1*A*). It is translated from the AUG initiation codon upstream of the core gene on the precore messenger RNA (mRNA), generating a product with an additional 29 amino acid residues on the amino terminus. This 25 k daltons precore/core fusion protein (p25) is post-translationally modified by signal peptidase and basic endopeptidase during the secretory pathway to generate a final circulating 15-17 k daltons HBeAg. HBeAg may promote immune tolerance during perinatal infection and alter host immune response against core protein.[3] The anti-HBe immune response plays a critical role in the clearance of HBV during natural infection. Thus, seroconversion from HBeAg to anti-HBe is usually associated with marked drop in viremia and normalization of aminotransferase levels. Continued expression of HBeAg at this stage of infection may be

detrimental to viral survival.

Two types of HBeAg variants, with reduction and termination of HBeAg expression, emerge sequentially at the late stages of HBV infection. Reduction in HBeAg expression by the core promoter mutants is a result of declined precore mRNA transcription,[4-7] while abolition of HBeAg expression in the precore mutants is achieved by nonsense or frameshift mutations in the precore region.[8-10] Both types of HBeAg variants are adaptive variants selected by the host anti-HBe immune pressure.

A unique feature of South African (SA) black carriers of HBV is that HBeAg expression is lost very early during the course of infection; only 5% are HBeAg positive in adulthood compared to a rate of 40% or higher found in other hyperendemic areas of the world such as Southeast Asia. The clinical impact of rapid HBeAg seroconversion on disease progression is still unclear. Recently, partial sequences of HBV genomes isolated from Black South African patients were

reported. Of particular interest was the finding that 80% of SA HBV strains harbor double or triple point mutations at nucleotide 1809, 1811, and 1812 of the HBV genome, the -5, -3, and -2 positions of the precore translation initiation codon,[11] which might impair HBeAg expression as a result of sub-optimal translation initiation.[12-14] This hypothesis was formally tested in the present study by site-directed mutagenesis and transfection experiments. If proven this, this finding may solve the mystery of accelerated seroconversion as found in South African patients. It may also reveal third mechanism whereby the virus can down-regulate HBeAg expression.

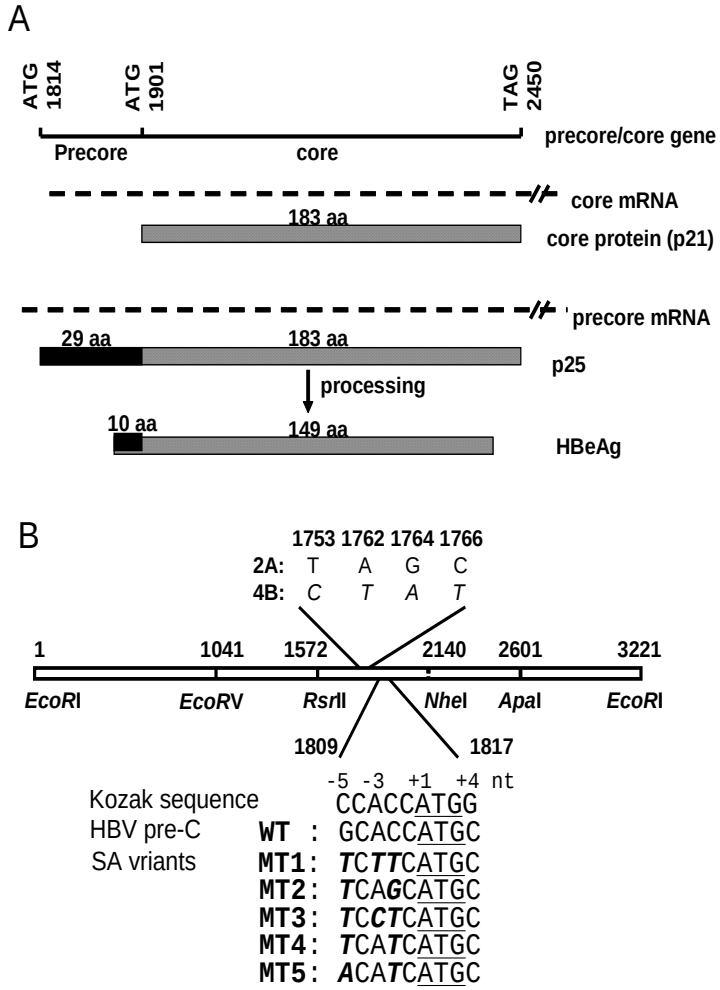


Figure 1. HBeAg biosynthesis and South African variations near the precore AUG codon. (*A*) A diagram on the expression of core protein and HBeAg. Usually no core protein expression takes place from precore mRNA. aa: amino acids. (*B*) The South African sequence variations and their introduction into the full-length HBV genome. The top panel shows some of the unique restriction sites present in 2A and 4B clones. The *Nhe*I site was artificially created so that the SA variants can be cloned into the *Rsr*II – *Nhe*I sites. Also shown are the difference in core promoter sequence between 2A and 4B clones. The lower panel depicts the five types of SA variants used in this study. They diverge from the Kozak sequence for optimal translation and also from consensus sequence of HBV genomes elsewhere. The precore ATG codon is underlined, and mutated nucleotides in the SA strains are shown in bold, italic.

II. Materials and Methods

1. Subjects

Blood was obtained, with informed consent, from 45 black Africans. Eighteen were HBsAg positive asymptomatic carriers with normal serum alanine aminotransferase levels. The HBeAg status of these patients is summarized in Table 1. Five patients suffered from acute hepatitis. Alanine aminotransferase levels in these patients ranged between 799 IU/L and 4747 IU/L. One patient with fulminant hepatitis was a female aged 32 years with an alanine aminotransferase level of 979 IU/L. In addition 21 children were studied. Fifteen of the children with biopsy proven HBV membranous nephropathy were index cases in a previous study[15] and 6 were family members of these index cases. All had normal alanine aminotransferase levels.

2. Sequencing

DNA was extracted from the serum samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the protocol provided by the manufacturer. In order to sequence

the region upstream of the precore translational start codon the region was amplified by nested PCR using primers 1687(+) and 2498R(-) in the first round and 1730(+) and 2043R(-) in the second round.[16] Sequencing of PCR products was performed manually using the Sequenase PCR product sequencing kit (Roche Diagonstics Co. Indianapolis, IN, USA) or automatically using Big Dye™ Terminator v3.0 Cycle sequencing kit (Applied Biosystems, Foster city, CA, USA) and the 377 DNA sequencer (Applied Biosystems, Foster city, CA, USA).

3. The parental HBV clones and site-directed mutants for transfection

Because the majority of SA isolates of HBV belong to genotype A,[11,17,18] we used two well-characterized HBV genomes of this genotype to create the SA mutations (Figure 1*B*). Clone 2A had wild-type core promoter sequence, thus mimicking viral genomes in the early HBeAg-positive phase of infection. Clone 4B was a high replicating core promoter mutant representing viral genomes in the late stage of infection. Use of both HBV constructs allowed us to examine the effect of the SA mutations

alone and in combination with core promoter mutations, which occur at the late stage of infection.[11]

(1). Serum samples and DNA extraction

Serum samples are collected from HBeAg positive chronic carriers infected with same genotype A. The serum samples are diluted in TEN buffer and digested at 37°C for 2 hrs with Proteinase K (0.5 mg/ml) in the presence of SDS (0.5%). DNA is extracted with phenol/ chloroform/ isoamyl alcohol (25:24:1) and precipitated with ethanol. Purified DNA is resuspended in water.

(2). PCR and cloning

Full-length HBV genomes are PCR amplified according to the method of Gunther et al[19] and cloned into pUC18 Vector. To facilitate the cloning of PCR products, both primer sequences are modified at the 5' end such that the sense primer contained just HindIII site (5' - CCG GAA AGC TTA TGC TCT TCT TTT TCA CCT CTG CCT AAT CAT C - 3', underlined) while the antisense primer contained SacI site only (5' - CCG GAG AGC TCA TGC TCT TCA AAA AGT TGC ATG GTG CTG GTG - 3', underlined).

Forty cycles of amplification are performed with the Expand high fidelity PCR system (Roche Diagonstics Co. Indianapolis, IN, USA) using conditions specified by Gunther et al.[19] The PCR products are cloned into the HindIII/SacI sites of pUC18 Vector.

(3). Site-directed mutagenesis

Point mutations were generated by the PCR-based overlap extension method,[20] using the high fidelity PCR system (Roche Diagonstics Co. Indianapolis, IN, USA) and 25 cycles of amplification for each PCR reaction. To further reduce the possibility of unwanted PCR errors, an artificial NheI site was introduced into position 2140 of both 2A and 4B genomes. The NheI mutations did not alter core protein sequence, and the corresponding dimers did not differ from the original 2A or 4B clones in terms of viral replication or gene expression (data not shown). The PCR products containing the SA mutations were digested with the RsrII/NheI restriction enzymes to replace the cognate fragment (position 1572 - 2140) in wild-type 2A and 4B clones. The entire PCR derived region was subsequently sequenced to confirm the presence of desired mutations and lack

of unwanted ones. For comparison of HBeAg expression with the common core promoter mutants, a 2A-based construct with $1762^{T}1764^{A}$ core promoter mutations (mu1) was employed. To evaluate the impact of hepatitis B virus X protein (HB_X) function on HBeAg expression, a C to G change was introduced into position 1706 of the 2A constructs with and without the SA mutations, creating a TAG stop codon in the HB_X gene.

(4). Tandem dimer construction

For HBV replication and gene expression to proceed under the control of endogenous core promoter, each construct was converted into a tandem dimer. To make head-to-tail tandem dimers, the HBV DNA insert will be released from pUC18 vector by SapI/BglI double digestion and circulized with T4 DNA ligase. Sequently, HBV DNA is linearized with EcoRI and ligated with EcoRI digested, dephosphorylated pUC18 DNA at insert: vector ratio of 15~20:1. Tandem HBV dimer screened with an oligonucleotide probe spanning the tail-to-head junction of HBV dimer as previously described.[21] The 30-mer oligonucleotide covered the EcoR I site of HBV genome and had

the sequence 5' - GGC CAT GCA GTG GAA TTC CAC WRC YTT CCA - 3' (W=A+T; R=A+G, Y=C+T).

4. Transfection and analysis of viral replication/gene expression

(1). Transfecton

The dimeric HBV DNA constructs were transfected into Huh7 and HepG2 human hepatoma cells using a calcium phosphate transfection kit (Promega, Madison, WI, USA), using 3.6 μg or 5.4 μg DNA per 6-cm dish. Cells were harvested and culture supernatant was collected at day 5 posttransfection. In order to reduce experimental variability, all the constructs were tested at least five times.

(2). Isolation of core particle- related HBV DNA

HBV replication was monitored by Southern blot analysis of HBV DNA associated with intracellular core particles.[22] HBV core particles will be obtatined from an aliquot of transfected cells basically as described.[22] Cells are lysed with 400μl of buffer containing 10 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM

EDTA, and 1% NP 40. The lysate is supplemented with 10 mM $CaCl_2$, 12 mM $MgCl_2$, and digested at 37°C for 15 min with DNase I (2 u) and mung bean nuclease (30 u) to degrade transfected HBV DNA. Core particles are precipitated with 150μl of 26% PEG solution (1.2 M NaCl, 60 mM EDTA, 30% sucrose, 26% PEG), resuspended in 100μl of solution containing 10 mM Tris, pH 7.5, 6 mM $MgCl_2$, and 8 mM $CaCl_2$, and digested with 2 u of DNase I and 3 u of mung bean nuclease at 37°C for 10 min to further remove transfected DNA. After addition of 270μl of protease digestion buffer (25 mM Tris, pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS), samples are digested at 37°C for 1 to 2 hr with proteinase K (0.5 mg/ml). DNA is extracted with phenol and precipitated with ethanol. Purified DNA is subjected to Southern blot analysis with a highly pure full-length HBV probe (obtained by two rounds of PCR amplication).

(3). Southern blot hybridization of HBV DNA

HBV DNA was resolved in 1.0% agarose gels, transferred to nylon membranes (Hybond N+; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) by Southern blotting, and

hybridized with an alkaline phosphatase- labeled wild-type full-length HBV DNA probe obtained by two rounds of PCR amplification. Gel electrophoresis was either performed in the absence of ethidium bromide or in its presence both in the gel and in the running buffer, which greatly accelerated migration of the single stranded HBV genome.

(4). HBsAg and HBeAg assay

The HBsAg secreted to culture supernatant is measured by an enzyme immunoassay (Auszyme kit; Abbott Laboratories, Abbott Park, IL, USA) using the protocol provided by the manufacturer. The samples were diluted 3 or more times with phosphate-buffered saline to avoid signal saturation. For those samples with OD at 492nm greater than 3, a two fold serial dilution is performed until the OD value fell within the readable range. Secreted HBeAg is detected by radioimmunoassay (EIA kit; DioSorin, Stillwater, MN, USA). The monoclonal antibody for HBeAg detection did not cross-react with Hepatitis B core antigen (HBcAg).

5. Core protein mediated trans-complementation of HBV replication

A replication deficient mutant of clone 4B was generated by a C to G change at position 2044, creating a TGA nonsense mutation in the core gene. As the potential source of the core protein, the entire precore/core gene of wild-type 4B and SA mutants (MT1, MT3) was amplified by PCR, using sense primer 5'- GGA GGC TCG AGG CAT AAA TTG GTC TGC GCA CC -3' (XhoI site underlined), and antisense primer 5'- AAA GCG AAT TCA AGT TTC CCA CCT TAT GAG TCC -3' (EcoRI site underlined). The PCR products were cloned into the XhoI/EcoRI sites of the pcDNA3.1 Zeo(-) vector, which contains a cytomegalovirus promoter to drive eukaryotic gene expression. When transcribed, the 5' end HBV sequence in the mRNA would correspond to the 5' end of the authentic precore mRNA. As a positive control of core protein expression, the core gene of wild-type 4B genome was amplified using the same antisense primer but a different sense primer 5'- AGC ACC TCG AGA CTT TTT CAC CTC TGC CTA ATC ATC -3' (XhoI site underlined). The precore and core constructs (3.6 μg DNA) were separately

co-transfected with the tandem dimer of core-minus 4B genome (3.6 μg DNA). Viral genome replication was analyzed from lysates of cells harvested at day 5 after transfection.

6. In vitro transcription / translation

The above-mentioned PCR primers were used to amplify the entire precore/core gene or the core region alone, using 2A-based constructs as templates. The PCR products were cloned into the XhoI/EcoRI sites of pBluescript SK vector. Coupled transcription/translation was performed at 30°C using a commercial kit (Promega, Madison, WI, USA) with rabbit reticulocyte lysates, [^{35}S]-methionine, and T7 polymerase. An aliquot of translation product was electrophoresed through a 12% polyacrylamide gel, and treated with enlightening solution (PerkinElmer, Boston, Massachusetts). Dried gels were exposed to X-ray films at – 80°C.

7. Statistical Analysis

The difference of the HBeAg/HBsAg levels of the wild-type and SA mutants was determined by Student's t-test. A value of $p < 0.05$ was taken to be statistically significant. All of

statistical analysis was performed using Window-SPSS release 10.0 for personal computer.

III. Results

1. The SA mutations are present throughout the course of infection

Many individuals described in a previous study had seroconverted to anti-HBe and contained core promoter mutations. A subset of these patients had developed hepatocellular carcinoma.[11] Thus, the possibility that sequence changes at 1809-1812 of the HBV genome represent adaptive change under immune pressure, similar to that observed with core promoter and precore mutations, could not be excluded. For this reason, HBV isolates from 6 acute hepatitis patients (one with fulminant hepatitis), 21 HBV infected children, and 18 asymptomatic carriers, most at the HBeAg-positive phase of infection, were sequenced. As shown in Table 1, all 6 acute HBV patients contained HBV with the $1809^{T}1812^{T}$ double mutation. In addition, 90% (19/21) of the isolates from children were found to

harbor the $1809^{T}1812^{T}$ double mutation. This prevalence is similar to that reported in our previous study.[11] Among the 18 asymptomatic carriers, 11 were infected with the SA subtype A' of genotype A,[17] 2 with conventional genotype A, and 5 with genotype D. All the genotype D and genotype A as well as 3 of the genotype A' isolates had wild-type sequence preceding the precore AUG codon. For the remaining genotype A' isolates, 5 contained the $1809^{T}1812^{T}$ double mutation and 3 contained the $1809^{T}1812^{G}$ double mutation. These latter 3 individuals, but none of the other 15 asymptomatic carriers, harbored $1762^{T}1764^{A}$ core promoter mutations. Thus, the 1809-1812 mutations are highly prevalent in HBV genotype A' infected South Africans with acute infection, during childhood, and at the HBeAg-positive phase of infection. These findings suggest the 1809-1812 mutations as stable traits of genotype A' isolates in South Africa, rather than adaptive mutations under anti-HBe immune pressure.

Table 1. Prevalence of 1809-1812 mutations in black South Africans.

	1809-1812 sequence	No. of patients	Age range	M / F	HBeAg[1] + / -
Acute hepatitis[2]	WT	0	18 – 32	-	-
	$1809^{T}1812^{T}$	6		3 / 3	3 / 0
Children[3]	WT	2	4 – 16	2 / 0	1 / 1
	$1809^{T}1812^{T}$	19		17 / 2	14 / 5
Asymptomatic carriers	WT	10[4]	20 - 40	10 / 0	6 / 0
	$1809^{T}1812^{T}$	5		5 / 0	3 / 1
	$1809^{T}1812^{G}$	3[5]		3 / 0	2 / 0

[1] Some samples not available for HBeAg assay

[2] One case with fulminant hepatitis

[3] Fifteen with membranous nephropathy

[4] Five infected with genotype D, 2 with genotype A, and 3 with A'

[5] All contained $1762^{T}1764^{A}$ core promoter mutations

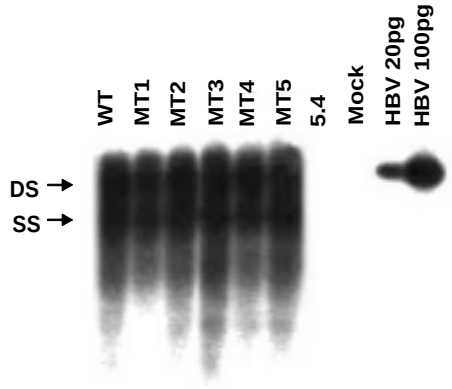
2. The SA mutations reduced HBeAg production in the context of replicating HBV genome

Five types of naturally occurring SA mutations identified in a previous study[11] were introduced into two HBV genomes of the same genotype (2A and 4B; Figure 1*B*). Of these, MT1 ($1809^{T}1811^{T}1812^{T}$) and MT3 ($1809^{T}1811^{C}1812^{T}$) contained triple mutations at the -5, -3, and -2 positions relative to the adenosine of the precore AUG codon, while MT2 ($1809^{T}1812^{G}$), MT4 ($1809^{T}1812^{T}$), and MT5 ($1809^{A}1812^{T}$) had double mutations at the -5 and -2 positions. An HBV clone (construct 5.4) defective in genome replication and HBeAg expression owing to a single nucleotide deletion in the core gene was included as a negative control. Following transfection of the tandem dimers of these constructs into Huh7 and HepG2 human hepatoma cells, HBeAg and HBsAg expression was analyzed. Figure 2 shows results of one typical experiment of 4B-based constructs in Huh7 cells. The various constructs did not differ significantly in the levels of intracellular core particle-associated DNA, or secreted HBsAg (panels *A* and *B*). These results suggest similar transfection efficiencies of the constructs because the mutations

preceding the precore translation initiation codon are not expected to modulate HBsAg expression.

HBeAg expression was greatly reduced by the MT1 and MT3 mutations, and also appears diminished by the MT4 and MT5, but not MT2 mutations (Figure 2*B*). To precisely evaluate the effects of different SA mutations and to minimize the contribution of variations in transfection efficiencies, we used the ratio of net HBeAg / net HBsAg values as an indicator of the efficiency of HBeAg expression (the net value is equal to the value of the sample minus the value obtained from mock transfected cells). Assuming a 100% efficiency of HBeAg expression by the parental 4B clone, the efficiencies for the MT1, MT2, MT3, MT4, and MT5 mutants were 17%, 102%, 24%, 82%, and 78% in this particular experiment (Figure 2*B* and 2*C*).

A



B

	WT	MT1	MT2	MT3	MT4	MT5	5.4
Net HBeAg *(cpm)*	928	155	1007	227	728	772	-12
Net HBsAg *(O.D.490nm)*	2.22	2.13	2.36	2.25	2.12	2.36	1.97
HBe/HBsAg ratio	418	73	427	101	343	327	-6
Efficiency (%)	100	17	102	24	82	78	-1

C

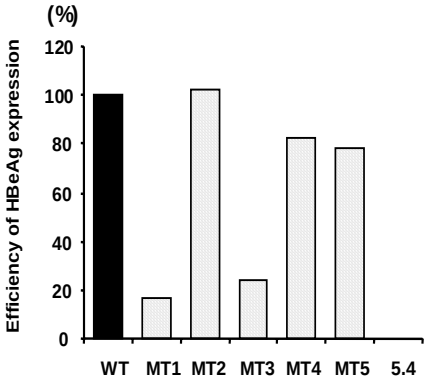
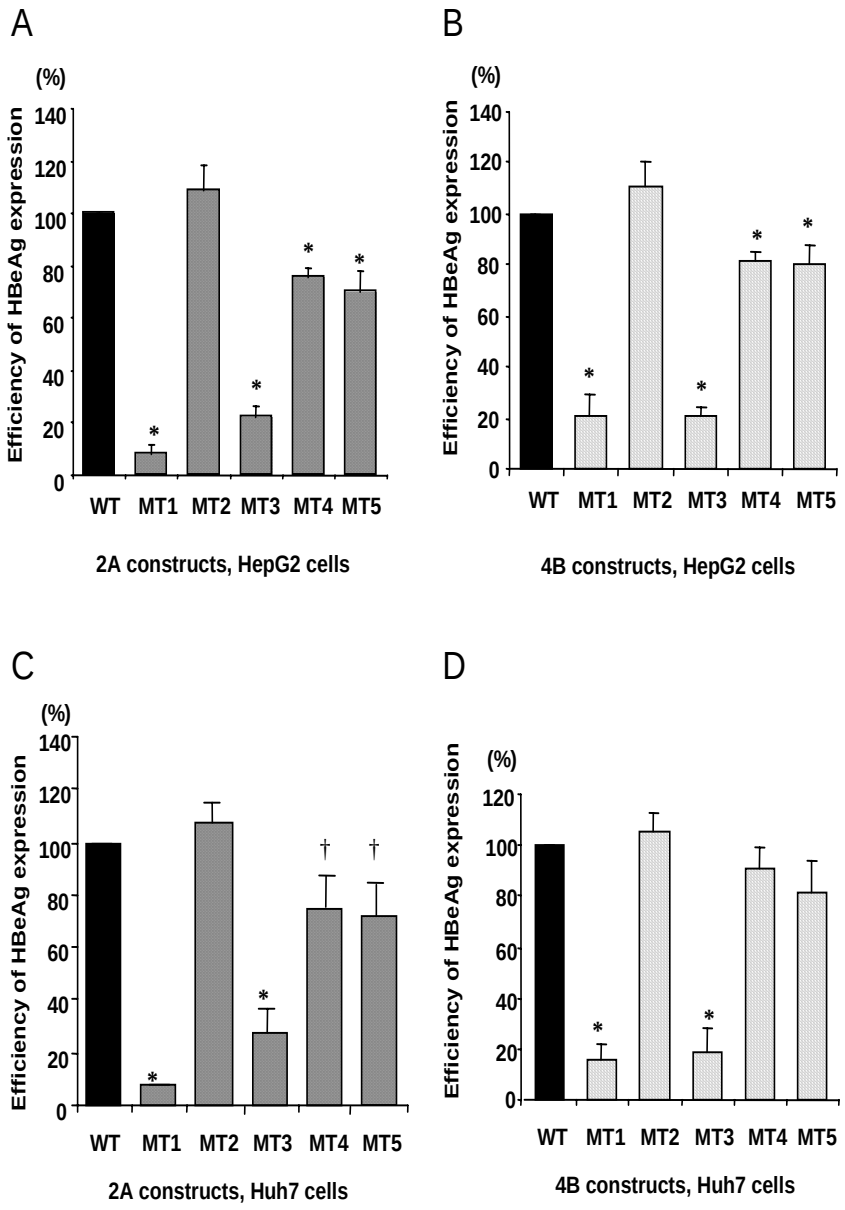


Figure 2. Results of one transfection experiment with 4B-based constructs in Huh7 cells. (*A*) HBV DNA replication inside core particles. DS: double stranded HBV DNA; SS: single stranded HBV DNA. (*B*) A procedure to calculate the efficiency of HBeAg expression. Net values of HBeAg and HBsAg were obtained by subtracting the values from supernatant of mock transfected cells. Clone 5.4 has a single nucleotide deletion in the core gene, hence negative for viral replication and HBeAg expression. (*C*) A graphic view of the efficiency of HBeAg expression.

Figure 3*A* summarizes results of transfection experiments using 2A-based constructs in HepG2 cells. The MT1 and MT3 triple mutations suppressed HBeAg expression to 8% and 28% of the wild-type levels, respectively, while the MT4 and MT5 double mutations reduced HBeAg expression to 74% and 72% of the wild-type levels, respectively. Similar results were obtained in Huh7 cells (Figure 3*C* and *D*). Transfection of Huh7 cells with 4B-based constructs resulted in a similar trend in the reduction of HBeAg levels except that the HBeAg levels were much lower relative to those produced by 2A-based constructs (Figure 3*C*). The $1753^{C}/1762^{T}/1764^{A}/1766^{T}$ quadruple core promoter mutation in clone 4B is known to greatly reduce HBeAg expression (data not shown). These results are compatible with the concept that the core promoter mutations and the 1809-1812 mutations affect HBeAg biosynthesis at different levels, and hence their effects may be additive.


A
(%)
Efficiency of HBeAg expression
140
120
100
80
60
40
20
0
WT MT1 MT2 MT3 MT4 MT5
2A constructs, HepG2 cells
B
(%)
Efficiency of HBeAg expression
140
120
100
80
60
40
20
0
WT MT1 MT2 MT3 MT4 MT5
4B constructs, HepG2 cells
C
(%)
Efficiency of HBeAg expression
140
120
100
80
60
40
20
0
WT MT1 MT2 MT3 MT4 MT5
2A constructs, Huh7 cells
D
(%)
Efficiency of HBeAg expression
120
100
80
60
40
20
0
WT MT1 MT2 MT3 MT4 MT5
4B constructs, Huh7 cells

E

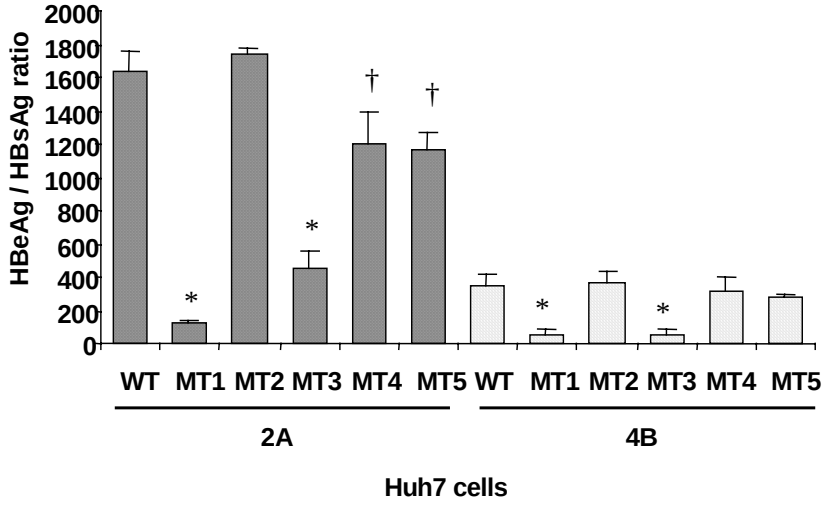


Figure 3. Summary of the effects of SA mutations on HBeAg expression from different genetic backgrounds (2A *vs*. 4B) and two hepatoma cell lines (Huh7 *vs*. HepG2). The results are shown in HBeAg / HBsAg ratios for panel *E*, and as percentiles of the wild-type genome for panels *A* to *D*. Statistical differences between the wild-type and SA mutations are shown. * : p <.001, † : p <.05 (vs. the wild-type clone 2A or 4B)

3. The SA mutation reduced HBeAg expression to a similar extent as the core promoter mutations

Of the five types of SA mutations tested in this study, the MT4 ($1809^{T}1812^{T}$) double mutation was the most common, accounting for more than 80% of the isolates found in South Africa[11] (Table 1). The effect of this double mutation on HBeAg expression was compared with that of the most common core promoter mutations ($1762^{T}/1764^{A}$, construct mu1). The MT4 mutant suppressed HBeAg expression to 67% of the wild-type level, while mu1 produced 70% of the wild-type level (Figure 4). Thus, the effect of the $1809^{T}1812^{T}$ mutation was similar to that of the core promoter mutations.

4. The reduction in HBeAg expression by the SA mutations was independent of HBx function

Because the HB_X gene overlaps with the core promoter and 5' precore region, mutations around precore AUG codon could modify C-terminal sequence of HBx. Indeed, all five SA mutations studied here induced double amino acid substitutions in the HBx (Figure 5*C*, inset). To establish whether sequence

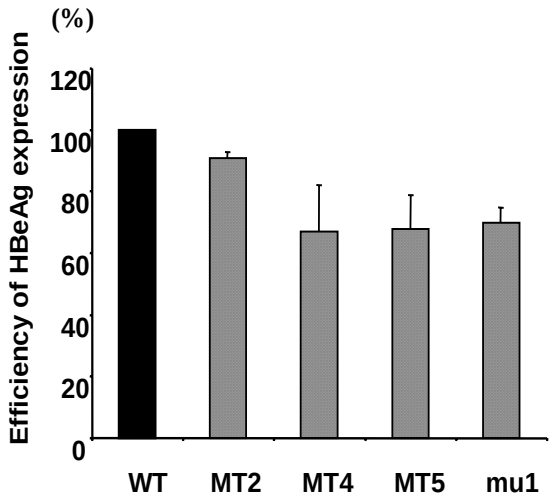


Figure 4. Comparison of SA double mutations with the $1762^{T}/1764^{A}$ core promoter mutations (mu1) on HBeAg expression. All the constructs shown here are 2A-based, and experiments were performed in Huh7 cells. The relative HBeAg expression efficiencies were 92%, 67%, 68%, and 70% for MT2, MT4, MT5, and mu1, respectively.

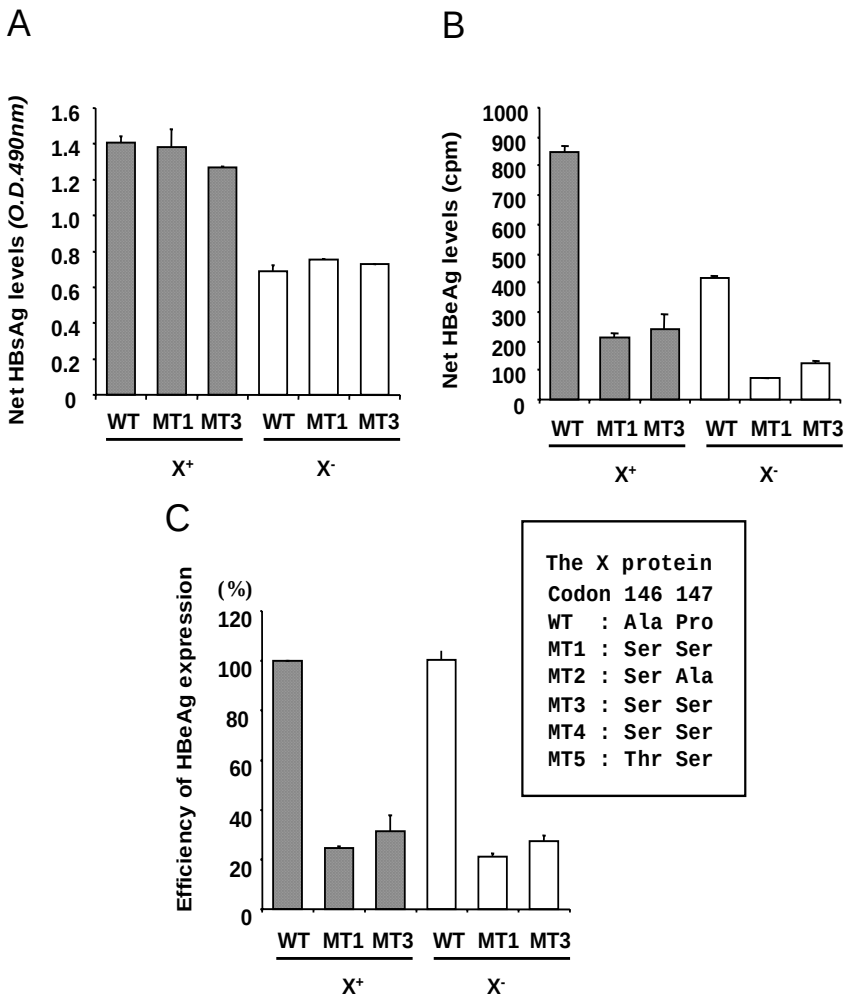


Figure 5. Effect of changes in HBx function on HBsAg and HBeAg expression in transfected HepG2 cells. HB_X-intact (X^+) and HB_X-defective (X^-) versions of WT, MT1, and MT3 constructs in 2A background were analyzed. (*A*) HBsAg levels. (*B*) HBeAg levels. (*C*) Efficiency of HBeAg expression. Amino acid changes in HBx are shown in the inset of panel *C*. Inactivation of HB_X function reduced both HBsAg and HBeAg expression for all the three constructs, but the MT1 and MT3 constructs continued to express much less HBeAg than their WT counterpart. DS: double stranded HBV DNA; SS: single stranded HBV DNA.

changes of HBx were responsible for the reduction in HBeAg expression, we abolished HBx expression by a nonsense mutation upstream of the SA mutations. Only the MT1 and MT3 mutants were studied, as they displayed the greatest reduction in HBeAg expression. Experiments in Huh7 cells did not reveal any effect of HB_X gene inactivation on HBsAg or HBeAg expression (data not shown). In HepG2 cells HB_X-minus mutants expressed roughly half the amount of HBsAg and HBeAg as compared with their HB_X-competent counterparts (Figure 5*A* and 5*B*). However, HBx-minus MT1 and MT3 constructs still produced far less HBeAg than the HBx-minus WT clone (Figure 5*C*), suggesting that amino acid changes in HBx were not responsible for the inhibition of HBeAg expression.

5. The SA mutations produce leaky scanning of the precore AUG codon

According to the scanning model, translation of most eukaryotic mRNAs is initiated by binding of the 40S ribosomes to the 5' cap structure, followed by scanning for the AUG with an optimal surrounding sequence.[23] Translation from this AUG codon will prevent initiation from other downstream AUG codons. The

precore AUG has a favorable context for translational initiation and thus only HBeAg, but no core protein, is expressed from the intact precore mRNA.[24-26] Because the critical -3 position of precore AUG[12,13] is mutated in the triple mutants from A to T (MT1) or C (MT3) (Figure 1*B*), reduced translational initiation was probably responsible for diminished HBeAg expression. In such instances, continued scanning of ribosomes down towards the core AUG codon may result in core protein expression. To test this hypothesis, we cloned the entire precore/core sequence of 2A, and 2A-based MT1 and MT3 constructs into the pBluescript vector, and performed *in vitro* transcription/translation experiments using T7 polymerase. While the precore/core sequence of wild-type 2A produced only p25 (primary translation product of the precore/core gene and the precursor to HBeAg, Figure 1*A*), the MT1 and MT3 constructs produced a 21 k daltons protein (p21) indistinguishable from core protein synthesized from core mRNA (Figure 6). In addition, the amount of p25 expressed from the MT1 and MT3 constructs was greatly reduced. These results confirmed a leaky scanning mechanism of the precore AUG in

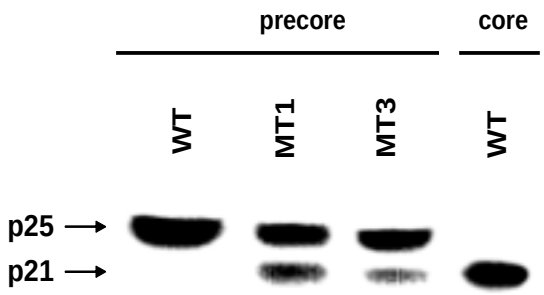


Figure 6. Molecular evidence for a leaky scanning mechanism with respect to translation from the precore mRNA of MT1 and MT3 variants. *In vitro* transcription and translation of the precore and core mRNA were performed in rabbit reticulocyte lysates. MT1 and MT3 produced less 25 k daltons protein (p25, HBeAg precursor) than the wild-type control and generated some 21 k daltons core protein (p21).

the triple SA mutants as the explanation for reduced HBeAg expression.

6. The replication defect of a core-minus HBV genome can be rescued by precore mRNA containing the triple SA mutations

A genetic complementation experiment was designed to confirm p21 expressed from the precore mRNA of MT1 and MT3 as the authentic core protein. During natural HBV infection the pregenomic RNA serves both as mRNA for core and polymerase expression, and as pregenome to be encapsidated into newly formed core protein particles, where replication takes place.[1] For transfection experiments in cell lines, it is possible to ablate core or polymerase protein expression from pregenomic RNA via a nonsense mutation. When a plasmid DNA capable of generating such a pregenomic RNA is co-transfected with another plasmid encoding the missing protein (core or polymerase), viral DNA replication will be rescued.[27] Introduction of a nonsense mutation into the core gene of clone 4B abolished viral replication, which could be rescued by co-transfection with the 4B core gene

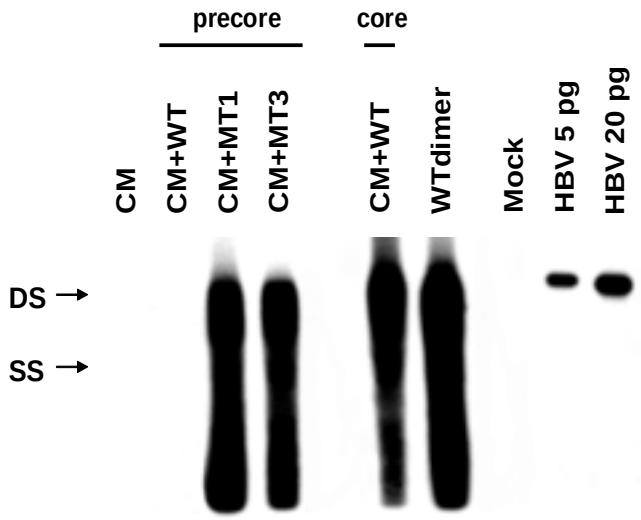


Figure 7. Functional evidence for a leaky scanning mechanism with respect to translation from the precore mRNA of MT1 and MT3 variants. Complementation of HBV replication by core protein provided in *trans.* The entire precore/core gene was amplified from 4B-based wild-type and MT1, MT3 constructs, and cloned into the pcDNA3.1 Zeo(-) vector. The core gene of 4B was also cloned into the same vector as a positive control. These constructs were co-transfected with the core-defective 4B dimer (CM). Viral replication was rescued by 4B core mRNA, and also by the precore mRNA harboring MT1 and MT3 mutations.

cloned into the pcDNA vector (Figure 7). Interestingly, co-transfection with the entire precore/core sequence also rescued the replication defect of the 4B dimer if the MT1 or MT3 mutations were present (Figure 7), thus confirming core protein production from the precore mRNA of the triple SA mutants.

IV. Discussion

The sequence around the precore initiation codon is conserved in all the eight known genotypes as 5' - AGCACCAUGC - 3' (nt 1808 – 1817).[28-31] This sequence conforms to the optimal context for translational initiation, the so-called Kozak sequence: 5' - GCC$^{A}/_{G}$CCAUGG - 3' .[13, 23] In contrast, many South African HBV strains, which belong to a subgroup of genotype A,[11,17,18] harbor point mutations immediately upstream of the precore AUG codon.[11] Such HBV variants have also been found occasionally in other parts of the world.[32-34] The most prevalent South African variant contained double nucleotide substitutions: 5' - *T*CA *T*CATGC - 3' (mutated nucleotides in italics, MT4 in Figure 1*B*), but variants

with triple nucleotide substitutions have also been detected, such as 5' - *T*C*TT*CATGC - 3' (MT1) and 5' - *T*C*CT*CATGC - 3' (MT3).[11] Site-directed mutagenesis followed by transfection experiments demonstrated several fold reduction in HBeAg expression by the MT1 and MT3 mutants (Figure 3). The common MT4 mutation and another double mutation (MT5) displayed 20% or more reduction in HBeAg expression, while the MT2 double mutation exhibited slightly enhanced HBeAg expression. Taken together, these results confirm the critical importance of an adenosine or guanine at the -3 position for translation efficiency,[13] and also suggest a modulating effect of changes in the -2 position.

The effect of the South African mutations on HBeAg expression was independent of that of the core promoter mutations, but the two sets of mutations had an additive effect (Figure 3*E*). Thus, while the core promoter mutations in clone 4B drastically reduced HBeAg expression, addition of the South African mutations further reduced HBeAg expression to a similar extent observed in the 2A background. The clinical significance

of this finding is that development of core promoter mutations in the SA variants, which does occur in this group of HBV strains,[11] will further reduce HBeAg expression. All the South African mutations caused double amino acid changes in the HBx (Figure 5), a viral transcriptional transactivator.[36] However, the three mutants with identical amino acid substitutions in HBx (MT1, MT3, and MT4) produced quite different levels of HBeAg upon transfection into either HepG2 or Huh7 cells (Figure 3). Introduction of a stop codon into the HBx gene upstream of the 1809-1812 mutations did not have any effect on HBeAg or HBsAg expression in Huh7 cells, which is consistent with the observation that the HB_X gene had a limited effect on HBV replication in this particular cell line.[37] On the other hand, blocking HBx expression in HepG2 cells reduced both HBsAg and HBeAg expression by about 50% (Figure 5*A* and 5*B*). This is in agreement with a previous study that showed that HBx expression is critical for HBV genome replication in HepG2 cells.[38] Nevertheless, the degrees in the reduction of HBsAg and HBeAg expression in MT1 and MT3 were similar to those found in the WT construct, which argues against the importance of

amino acid changes in the HBx on HBV gene expression.

According to the scanning model of mammalian gene translation, the 40S ribosomal subunits bind initially at the capped 5' terminus and scan down the mRNA sequence until an AUG with an optimal context is encountered, where translation is initiated.[23] When the sequence context of the first AUG is sub-optimal, a fraction of the 40S ribosomes will continue scanning and initiate translation from a downstream AUG codon (leaky scanning). The precore AUG codon has an optimal context for translational initiation and prevents core protein expression.[24-26] If the reduction in HBeAg expression by the South African mutations is mediated by sub-optimal context for initiation, then a substantial fraction of the 40S ribosomes will pass the precore AUG and initiate translation from the core AUG. This hypothesis was confirmed in this study by the *in vitro* transcription and translation experiments of the entire precore/core gene, which produces an mRNA species with a similar structure as the 5' end of the authentic precore mRNA. Indeed, we were able to observe reduced expression of p25 (HBeAg precursor) from the

MT1 and MT3 constructs, and detect de novo p21 expression from these two constructs but not from the wild-type 2A (Figure 6). The identity of this molecule as authentic core protein was confirmed by a functional assay, where precore mRNA containing the triple mutations rescued the replication defect of a core-minus 4B dimer mutant (Figure 6). However, we could not demonstrate above findings with double point mutations (MT2, MT4, MT5) using *in vitro* transcription/translation and trans-complementation experiments (Data was not shown). Probably these methods were not appropriate to detect smaller amount of HBeAg reduction than triple point mutations.

What is the functional consequence of reduced HBeAg expression? Black Africans predominantly contract HBV horizontally between the ages of 1 and 5 years and seroconvert to anti-HBe at a much earlier age than Southeast Asian patients. We hypothesize that reduced HBeAg expression as a result of the novel mutations upstream of the precore translation start codon will facilitate seroconversion to anti-HBe. In support of this concept, all patients with MT1 and MT3 mutations in a

previous study were already seroconverted to anti-HBe positive.[11] Although the most common MT4 mutation reduced HBeAg production to about 20-30% of wild-type virus and substantially less than the triple mutations (Figure 3), the reduction is comparable to that found with the common core promoter mutations ($1762^{T}/1764^{A}$) (Figure 4). It should be emphasized that both core promoter and precore mutations arise around or following seroconversion to anti-HBe, and therefore may be the result of the anti-HBe immune pressure rather than playing a direct role in seroconversion. On the other hand, the 1809-1812 mutations are present in children and at the acute stage of HBV infection (Table 1), and therefore may have a major effect in accelerating seroconversion in this population.

In summary, we have identified a novel class of mutations immediately preceding the precore AUG codon of South African HBV isolates that are present throughout infection. These HBeAg variants reduce HBeAg expression by a leaky scanning mechanism, and may facilitate the early HBeAg seroconversion seen in South African carriers of the virus, which is accompanied

by a striking reduction in viral replication and improvement in the necro-inflammatory activity of the liver.

V. Conclusions

Black South African (SA) carriers of hepatitis B virus (HBV) have been seroconverted from HBeAg to anti-HBe much earlier than do Asian patients. Since majority of SA HBV strains harbor point mutations immediately upstream of the precore AUG codon, the effect on HBeAg expression was investigated in this study.

The results as below were obtained:

1. The SA sequence changes were easily detectable in the acute, HBeAg positive phase of infection, suggesting they were not induced by immune pressure.
2. The SA mutations, especially the triple mutations, reduced HBeAg production in the context of replicating HBV genome.
3. The common MT4 double mutations reduced HBeAg

expression to a similar extent as the common core promoter mutations at 1762/1764.

4. Reduction of HBeAg expression by the SA mutations was additive with core promoter mutations, independent of X protein function, and mediated by ribosomal leaky scanning.
5. The SA strains of HBV thus represent a novel class of HBeAg variant which express less HBeAg due to sub-optimal context for translational initiation.
6. Combined with the clinical data, it is tempting to propose that SA mutations contribute to the accelerated seroconversion in South African patients.

South African mutations enabled core protein translation from precore mRNA, suggesting ribosomal leaky scanning of the precore AUG codon. Reduced HBeAg translation may contribute to earlier seroconversion to anti-HBe in this region of the world, and provide the molecular basis for a previously unexplained clinical event associated with viral clearance.

References

1. Ganem D, Schenider R. Hepatitis B virus. In: Knipe D. and Howley P, eds. Fields Virology. 4th ed. New York: Raven, 2001:2923-2970.

2. Magnius L, Espmark J. New specificities in Australia antigen positive sera distinct from the Le Bouvier determinants. J Immunol 1972;109:1017-1021.

3. Milich D, Jones J, Hughes J, Price J, Raney A, McLachlan, A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proc Natl Acad Sci USA 1990;87:6599-6603.

4. Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 1994;68:8102-8110.

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

6. Moriyama K, Okamoto H, Tsuda F, Mayumi M. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology 1996;226:269-280.

7. Scaglioni P, Melegari M, Wands J. Biological properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. Virology 1997;233:374-381.

8. Carman W, Jacyna M, Hadziyannis S, Karayiannis P, McGarvey M, Makris A et al. Mutation preventing

formation of the hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 1989;2:588-591.

9. Tong S, Li J, Vitvitski L, Trepo C. Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. Virology 1990;176:596-603.

10. Santantonio T, Jung M, Miska S, Pastore G, Will H. Prevalence and type of pre-C HBV mutants in anti-HBe positive carriers with chronic liver disease in a highly endemic area. Virology 1991;183:840-844.

11. Baptista M, Kramvis A, Kew MC. High prevalence of 1762T 1764A mutations in the basic core promoter of hepatitis B virus isolated from Black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology 1999;29:946-953.

12. Kozak M. Point mutations define a sequence flanking the

AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 1986;44:283-292.

13. Kozak M. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J Mol Biol 1987;196:947-950.

14. Kramvis A, Kew MC. The core promoter of hepatitis B virus. J Viral Hepatitis 1999;6:415-427.

15. Bhimma R, Coovadia H, Ramjee G, Kramvis A, Adhikari M, Kew MC et al. Characterization of proteinuria in asymptomatic family members and household contacts of children with hepatitis B virus-associated membranous nephropathy. Am J Kidney Dis 2001;37:125-133.

16. Owiredu W, Kramvis A, Kew MC. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. J Med Virol 2001;65:485-492.

17. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719-1729.

18. Kramvis A, Weitzmann L, Owiredu W, Kew M. Analysis of the complete genome of subgroup A' hepatitis B virus from South Africa. J Gen Virol 2002;83:835-839.

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

20. Ho S, Hunt H, Horton R, Pullen J, Pease L. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989;77:51-59.

21. Tong S, Li J, Vitvitski L, Benjelloun S, Trepo C. Rapid screening for bacterial colonies harbouring tandem hepatitis B virus sequences by an oligonucleotide probe. J Virol 1991;32:109-114.

22. Tong S, Li J, Vitvitski L, Trepo C. Replication capacities of natural and artificial precore stop codon mutants of hepatitis B virus: relevance of pregenome encapsidation signal. Virology 1992;191:237-245.

23. Kozak M. Initiation of translation in prokaryotes and eukaryotes. Gene 1999;234:187-208.

24. Weimer T, Salfeld J, Will H. Expression of the hepatitis B virus core gene in vitro and in vivo. J Virol 1987;61:3109-3113.

25. Jean-Jean O, Levrero M, Will H, Perricaudet M, Rossignol J. Expression mechanism of the hepatitis B virus (HBV) C gene and biosynthesis of HBeAg antigen. Virology

1989;170:99-106.

26. Tong S, Brotman S, Li J, Pascal D, Prince A, Trepo C. In vitro and in vivo replication capacity of the precore region defective hepatitis B virus variants. J Hepatol 1991;13(Suppl. 4): S68-S73.

27. Lavine J, Hirsch R, Ganem D. A system for studying the selective encapsidation of hepadnavirus RNA. J Virol 1989;63:4257-4263.

28. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo R, Imai M, Miyakawa Y et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69:2575-2583.

29. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;198:489-503.

30. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi R et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.

31. Arauz-Ruiz P, Norder H, Robertson B, Magnius L. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059-2073.

32. Estacio R, Chavez C, Okamoto H, Lingao A, Reyes M, Domingo E et al. Nucleotide sequence of a hepatitis B virus genome of subtype adw isolated from a Philippino: comparison with the reported three genomes of the same subtype. J Gastro Hepatol 1988;3:215-222.

33. Kidd-Ljunggren K, Oberg M, Kidd A. The hepatitis B virus X gene: analysis of functional domain variation and gene phylogeny using multiple sequences. J Gen Virol

1995;76:2119-2130.

34. Kidd-Ljunggren K, Oberg M, Kidd A. Hepatitis B virus X gene 1751 to 1764 mutations: implications for HBeAg status and disease. J Gen Virol 1997;78:1469-1478.

35. Laskus T, Rakela J, Steers J, Wiesner RH, Persing D. Precore and contiguous regions of hepatitis B virus in liver transplantation for end-stage hepatitis B. Gastroenterology 1994;107:1774-1780.

36. Koike K, Takada S. Biochemistry and functions of hepatitis B virus X protein. Intervirology 1995;38:89-99.

37. Blum H, Zhang Z, Galun E, von Weizsacker F, Gerner B, Liang T, Wands J. Hepatitis B virus X protein is not central to the viral life cycle in vitro. J Virol 1992;66:1223-1227.

38. Michael J, Bouchard M, Wang L, Schneider R. Calcium

signaling by HBx protein in hepatitis B virus DNA replication. Science 2001;294:2376-2378.

국문요약

Precore영역 번역개시 코돈 (AUG codon) 주위의 돌연변이가 B형 간염 바이러스 e 항원 (HBeAg) 발현에 미치는 영향

아시아와 아프리카 B형 간염 환자들은 대부분 수직감염이라는 같은 경로로 감염된다. 아시아인들과 달리 아프리카 흑인 환자들은 성인에 이르면 95%정도까지 B형 간염바이러스 e 항원(HBeAg)에서 항체(anti-HBe)로 혈청전환을 하는데 그 기전은 알려진 바 없다. 최근 남아프리카 흑인 환자들의 염기서열에서 80%이상이 precore 영역 번역개시 코돈 주위의 돌연변이가 일어났다고 보고된 바 있다. 본 연구는 아프리카 흑인들의 돌연변이가 HBeAg 발현에 미치는 영향과 기전을 규명하여 혈청전환이 빠르게 일어나는 이유를 알아보고자 하였다.

항원발현과 복제능의 변화를 조사하기 위하여 남아프리카 환자들에서 보여지는 5가지의 precore영역 번역개시 코돈 주위 돌연변이들을 같은 genotype A의 B형 간염 바이러스에 삽입시키고 trans-complementation과 *in vitro* translation 실험을 통해 기전을 규명하였다. 또한 45명의 남아프리카 흑인 환자들의 혈청을 조사, 비교하였다.

1. Precore영역 번역개시 코돈 주위의 염기서열 변이들은 HBeAg 발현을 억제시켰고 번역개시 코돈으로부터 - 3 위치의 변이가 가장 중요하였다.
2. 남아프리카 흑인 환자들에서 가장 흔한 $1809^{T}1812^{T}$ 변이는 - 3 위치의 변이가 없지만 기존에 알려진 가장 흔한 core promoter

변이($1762^{T}/1764^{A}$)와 비슷한 정도로 HBeAg 발현을 억제시켰다.

3. Precore영역 번역개시 코돈 주위의 변이들은 core promoter변이가 동반되었을 때 상합적인 영향을 보여주었고 B형 간염 바이러스 X 단백질의 변화와는 무관하였다.
4. *In vitro* translation과 Trans-complementation실험은 각각 분자생물학적과 기능적으로 precore영역 번역개시코돈 주위의 변이들이‘ ribosomal leaky scanning’ 이란 새로운 기전으로 HBeAg 발현을 억제하는 것을 입증하였다.
5. 남아프리카의 precore영역 번역개시 코돈 주위의 변이들은 주로 소아 또는 급성기의 간염에서 나타나 이 변이가 면역압력에 의해 나중에 발생한 것이 아니라 감염 초기부터 존재하였다는 것을 알 수 있었다.

이상의 결과에서 남아프리카의 precore영역 번역개시 코돈 주위의 염기서열 변이들은 ribosomal leaky scanning 기전으로 HBeAg 발현을 억제하였다. 이는 남아프리카인이 B형 간염바이러스의 수직감염 후 HBeAg 혈청전환이 빠르게 일어나는 분자생물학적 근거로 생각된다.

핵심되는 말 : B형 간염 바이러스, B형 간염 바이러스 e 항원, 남아프리카인, precore 및 core promoter 변이종, 번역개시코돈, 리보좀 리키스캔, 혈청전환