ANALYSIS OF MUTATIONS IN THE *S* GENE, CORE PROMOTER AND PRECORE GENE OF THE THAI ISOLATES OF HEPATITIS B VIRUS

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Hepatitis B virus is a major causative agent of hepatitis that can cause life-long chronic infection, cirrhosis, liver cancer, liver failure and death. Detection of viral and immunological markers are useful in the diagnosis and monitoring of the infection. The presence of HBeAg is one indicator of active viral replication, whereas anti-HBe antibody is believed to be a marker of recovery. However, some patients may have active viral infection in the absence of HBeAg which is postulated to be due to mutation in the genes encoding the HBc/HBe antigen.

In order to investigate the prevalence and importance of gene mutations in this region, nucleotide sequences were obtained from 61 chronic hepatitis patients. Of these 61 samples, genotype A, B and C were found in 1.6, 29.5 and 68.9%, respectively. Mutations in the core promoter region at position 1762 (A1762T) and 1764 (G1764A) were found in 33 patients (54.1%), in which 13 (39.4%) were HBeAg-negative and the other 20 (60.6%) were HBeAg-positive.

In the precore region, the classical mutation at position 1896 (G1896A) was found in 19 (31.1%) patients in which 17 (89.5%) were HBeAg-negative and the other 2 (10.5%) were HBeAg-positive. There is a significant correlation between HBeAg-negative status and mutation at G1896A ($p< 0.0001$). The mixed infection with wild type and mutant virus (G1896r) were found in 7 (11.5%) patients in which 6 (85.7%) were HBeAg-positive and one (14.3%) was HBeAg-negative.

In addition, the nucleotide sequences in the *S* gene were obtained from 10 patients; 8 were from genotype C, 2 were from genotype B. The sequences were used to design a synthetic peptide spanning the 'a' determinant of the HBsAg. However, attempts were unsuccessful in using this peptide as an immunodiagnostic reagent.

In the situation in Thailand where about almost 50 % of HBV infected patients had mutant viruses, HBeAg in the blood may not directly correlate with the absence of active hepatitis B infection.

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เกรียงศักดิ์ ฤชุศาศวัต: การศึกษาการเปลี่ยนแปลงรหัสพันธุ์กรรมส่วน *S* gene, core promoter และ precore gene ของเชื้อไวรัสตับอักเสบบีสายพันธุ์ไทย (ANALYSIS OF MUTATIONS IN THE *S* GENE, CORE PROMOTER AND PRECORE GENE OF THE THAI ISOLATES OF HEPATITIS B VIRUS) คณะกรรมการควบคุมวิทยานิพนธ์ : สิริฤกษ์ ทรงศิวิไล, พ.บ., Ph.D., ธารารัชต์ ธารากุล, พ.บ., Ph.D. 114 หน้า. ISBN 974-664-060-7

เชื้อไวรัสตับอักเสบบี เป็นสาเหตุสำคัญอย่างหนึ่งที่ก่อโรคตับอักเสบ ซึ่งทำให้เกิดการติดเชื้อแบบเรื้อรัง ตับแข็ง มะเร็งตับ และตาย

การตรวจหาการติดเชื้อของไวรัสตับอักเสบโดยใช้วิธีทางวิทยาภูมิคุ้มกัน สามารถช่วยการวินิจฉัยโรคได้อย่างถูกต้องและรวดเร็วมากขึ้น ในการติดเชื้อแบบเรื้อรังที่มีการตรวจพบแอนติเจนอี มีความสัมพันธ์กับการเพิ่มจำนวนของเชื้อไวรัส และการตรวจพบแอนติบอดีต่อแอนติเจนอี เชื่อว่ากำลังจะหายจากโรค แต่พบว่าผู้ป่วยบางรายที่มีแอนติบอดีต่อแอนติเจนอี สามารถตรวจพบ HBV DNA และมีอาการรุนแรงมากขึ้น ซึ่งสันนิฐานว่าน่าจะเกี่ยวข้องกับการเปลี่ยนแปลงของยีนที่สร้างแอนติเจน ส่วนคอร์และอี

การศึกษารหัสพันธุกรรมในบริเวณส่วน core promoter และ precore gene ของเชื้อไวรัสตับอักเสบบี ที่แยกได้ในกลุ่มผู้ป่วยโรคตับอักเสบเรื้อรัง จำนวน 61 ราย สามารถจำแนกเป็นสายพันธุ์ A, B และ C คิดเป็นร้อยละ 1.6 29.5 และ 68.9 ตามลำดับ ความเปลี่ยนแปลงในส่วน core promoter ที่ตำแหน่ง A1762T และ G1764A ในผู้ป่วยจำนวน 33 ราย คิดเป็นร้อยละ 54.1 ซึ่งสัมพันธ์กับการตรวจไม่พบแอนติเจนอี จำนวน 13 ราย (ร้อยละ 39.4) และตรวจพบแอนติเจนอี จำนวน 20 ราย (ร้อยละ 60.6)

ในส่วน precore gene การเปลี่ยนแปลงที่ตำแหน่ง G1896A มีจำนวน 19 ราย คิดเป็นร้อยละ 31.1 และสัมพันธ์กับผู้ป่วยที่ตรวจไม่พบแอนติเจนอี จำนวน 17 ราย (ร้อยละ 89.5) และให้ผลที่ตรวจพบ จำนวน 2 ราย (ร้อยละ 10.5) นอกจากนี้ยังพบการติดเชื้อแบบผสม ทั้งแบบดั้งเดิมและกลายพันธุ์ (G1896r) จำนวน 7 ราย คิดเป็นร้อยละ 11. 5 โดยผู้ป่วยส่วนใหญ่พบแอนติเจนอี 6 ใน 7 ราย (ร้อยละ 85.7) ไม่พบแอนติเจนอี 1 ใน 7 ราย (ร้อยละ 14.3)

การหาลำดับนิวคลิโอทัยด์ในส่วน *S* gene ที่มาจากผู้ป่วยชาวไทย จำนวน 10 ราย พบว่าเป็นสายพันธุ์ A จำนวน 8 ราย และสายพันธุ์ B จำนวน 2 ราย ส่วนของลำดับนิวคลิโอทัยด์ที่ได้ใช้เป็นแบบในการสังเคราะห์เส้นเปปไทยด์ให้คล้ายส่วนของ 'a' ซึ่งใช้เป็นแอนติเจนเคลือบเพลทในการตรวจหาแอนติบอดีโดยวิธีอีไลซ่า แต่อย่างไรก็ตามวิธีนี้ไม่สามารถใช้ในการตรวจหาแอนติบอดีได้

ผลการศึกษาครั้งนี้พบว่าสถานการของการติดเชื้อไวรัสที่พบในประเทศไทย ประมาณร้อยละ 50 มีการติดเชื้อไวรัสมิวเตนท์ ซึ่งทำให้การตรวจหาแอนติเจนอี อาจจะไม่สัมพันธ์กับการเพิ่มจำนวนของเชื้อไวรัส

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LIST OF ABBREVIATIONS

Abbreviation	Term
Ab	Antibody
ALT	Alanine transaminase
bp	Base pair
°C	Degree Celcius
cDNA	Complementary deoxyribonucleic acid
cccDNA	Covalently closely circular DNA
cm	Centrimeter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanidine triphosphate
dTTP	Deoxythymine triphosphate
DRs	Direct repeat sequence
DEPC	Diethylpyrocarbonate

LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
gm	Gram
gp	Glycoprotein
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HBeAg	Hepatitis B e antigen
HBcAg	Hepatitis B core antigen
kb	Kilobase
kDa	Kilodalton
LMP	Low melting point
M	Molar
mM	Millimolar
mg	Milligram
ml	Millilitre
mg	Microgram
µl	Microlitre

LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmole	picomole
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Taq	*Thermus aquaticus*
TMB	3,3´,5,5´ tetramethyl benzidine dihydrochloride
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

Hepatitis B infection is a serious disease caused by a virus that attacks the liver. Hepatitis B virus (HBV) can cause life-long infection, cirrhosis (scarring) of the liver, liver cancer, liver failure and death.

In the course of HBV infection, the presence of HBeAg is generally correlated with active viral replication and often active liver disease. On the other hand, the clearance of HBeAg and subsequent rise of anti-HBe antibody indicates the termination of HBV replication with or without integration of HBV DNA, and remission of liver disease (1). However, some patients have persistent viremia and severe active liver disease even after the clearance of HBeAg and these patients appears to be related to the continued replication and secretion of HBV (2, 3).

HBV mutations resulting in the inability to produce HBeAg often become the dominant viral quasispecies in the viral population present in the infected individuals. The mutations associate with the loss of HBeAg production frequently generate the stop codons or frameshift mutation within the precore (preC) leader sequence (4-10).

In addition to the most widely known mutation in the precore region or the 'classical mutation', analyses of the mutation occurring in the basal core promoter region (BCP) of Japanese patients with fulminant hepatitis revealed a correlation between HBeAg-negative phenotype and double mutation of A to T at nucleotide 1762 (A1762T) and G to A at nucleotide 1764 (G1764A). The implication of this observation was that this double mutation in the BCP could also prevent HBeAg expression (11).

In contrast to this finding (A1762T ,G1764A), Laskus *et al* examined the BCP sequence in HBV infected individual from the United States and found that while this double mutation was frequently observed, it was not correlated with HBeAg status of patients (12). By studying another cohort of Japanese patients, Takahashi *et al* suggested that this double mutation suppressed but did not abolish HBeAg expression and hence have termed this mutation an e-suppressive mutation. Thus there is a discrepancy concerning the effect of the BCP double mutations on the production of HBeAg (13).

There is little information on the mutation in the basal core promoter region, or the precore region in the patients in Southeast Asia which has very high prevalence of HBV infection.

This study was designed to study the role of the core promoter sequence changes in the apparent loss of HBeAg from the circulation, to analyze the pattern of the mutations such as stop mutation and frameshift mutation in with the precore sequence, and to analyze the possible association between the core promoter region or precore region mutations and the HBeAg/anti-HBe status.

CHAPTER II

OBJECTIVE

The objectives of this study are

1. To analyze the nucleotide sequence and the deduced amino acid sequence in the core promoter region and precore region of the Thai HBV isolates.

2. To analyze the correlation of these nucleotide sequence variations with the HBeAg status in HBV infected Thai patients.

3. To analyze the nucleotide sequence and the deduced amino acid sequence in *S* gene of the Thai isolates of HBV and to use the information to develop an enzyme immunoassay system for detecting anti-HBs antibody by using synthetic peptide designed from the sequence of the Thai isolates of HBV.

CHAPTER III

LITERATURE REVIEW

1. Hepatitis B Virus

Viral hepatitis is a systemic infection affecting the liver. At least 6 different human hepatitis viruses have been recognized and characterized in detail; hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV). The seventh agent has just been discovered and named as TT virus (TTV). All human hepatitis viruses are RNA viruses, except HBV and TTV which are DNA viruses. Despite the differences in their genome, molecular structure and virus classification, these hepatitis viruses all primarily target the liver cells and cause a necroinflamatory process, hepatitis. While all of them cause acute hepatitis, but only HBV, HDV and HCV can cause chronic hepatitis. The clinical outcomes of HGV and TTV infection have not yet been defined. Hepatitis viruses differ widely not only in their molecular structure but also in their modes of transmission and clinical features.

One of most important discovery in modern era of hepatitis research was the recognition by Blumberg and colleagues of Australia (Au) antigen. Subsequently, Prince and colleagues recognized the Au antigen (now known as the hepatitis B surface antigen or HBsAg) which was specifically found in the serum of patients with transfusion-associated hepatitis (14). In 1973, Dane found the virus-like particles in the sera of these patients. These particles were considered to be the hepatitis B virus (HBV) (15).

2. Clinical Manifestations of Hepatitis B Virus Infection

The incubation period for hepatitis B infection ranges from 45 to 180 days, and the onset is generaly insidious. The clinical presentation of acute hepatitis B infection is similar to that of HAV infection but tends to be more severe and may be associated with serum sickness-like syndrome. The clinical syndromes include vomiting, nausea, malaise, jaundice and increasing of serum transaminase levels. Although the hallmark of hepatitis is often jaundice, most initial infection caused by HBV produces non-icteric disease. The mildest attack is asymptomatic which is detectable only by an increase of the serum transaminase levels. Alternatively, the patient may be anicteric but suffer from gastrointestinal and influenza-like symptoms. The severity of infection may vary from asymptomatic infection to icteric, fulminant or fatal viral hepatitis.

Fulminant hepatitis is a rare form of the disease which usually overwhelms the patients within 10 days. This severe form of infection may develop so quickly that jaundice is unseen and may be confused with acute psychosis or meningo-encephalitis. On the other hand, the patients may become deeply jaundice. The warning signs may be repeated vomiting, a 'mousy' odor of the breath (fetor hepaticus), confusion and drowsiness. These are supervened by coma, indicating acute liver failure. The frequency of the fulminant course varies, depending on the type of viral hepatitis and prevalence of hepatitis B carriage but the evidence has also been unclear (16).

Most acute HBV infections result in complete recovery. However, there are some individual who are infected with HBV and subsequently develop chronic infection. Chronic hepatitis infection is represented by a spectrum of disease entities classified as chronic persistent hepatitis (CPH), chronic active hepatitis (CAH), and asymptomatic carrier state. Individuals with chronic HBV infections are usually asymptomatic carriers but patients with chronic hepatitis are at high risk for developing chronic sequelae and may die prematurely from either cirrhosis or liver cancer.

In chronic persistent hepatitis, hepatomegaly and splenomegaly could be detected but jaundice may or may not occurred. Liver dysfunction is most often demonstrated by recurrent hyperbilirubinemia and persistent of serum alanine transaminase (ALT) and aspartate transaminase (AST). In chronic active hepatitis, jaundice may or may not occurred. Elevation in serum transaminase enzymes (ALT and AST) accompanies the necrosis of liver cells that follows an inflammatory response.

Hepatocellular carcinoma (HCC), liver cancer, develops after a long time in individuals suffering from chronic hepatitis B infection. What events trigger the development of this disease form are currently unknown.

3. Epidemiology

Hepatitis B virus is globally distributed. The hepatitis B virus (HBV) infects more than 2000 million people alive today and 350 million persons are chronically infected carriers of the virus and are at high risk of death from active hepatitis, cirrhosis and primary hepatocellular cancer (17). Each year approximately 1 million people die from the acute and chronic sequelae of HBV infection, making it one of the major causes of morbidity and mortality in man.

As shown in Figure 1, approximately 45% of the global population live in areas with a high prevalence of chronic HBV infection (>8% of the population is HBsAg-positive); 43% in areas with a moderate prevalence (2-7% of the population is HBsAg-positive); and only 12% in areas with a low prevalence (<2% of the populations is HBsAg-positive). In high prevalence areas, including Thailand (18), the lifetime risk of HBV infection is more than 60% and most infections are acquired at birth or during early childhood when the risk of developing chronic infection is greatest. Approximately 35-75% of primary hepatocellular carcinoma in adults are HBsAg-positive (18-22).

4. Etiology

Hepatitis B virus (HBV) is the prototype member of genus *Orthohepadnavirus*, family *Hepadnavidae*. The defining features of the family are (1) relaxed circular, partially duplex DNA genomes of approximately 3 kb; (2) 40 diameter virion with an outer lipoprotein envelope (bearing surface protein) and an inner nucleocapsid or core (bearing the viral genome); (3) a virion-associated polymerase activity in the viral core; (4) production and export of 20 or 22 nm subviral lipoprotein particles composed of enveloped protein in the absence of other viral components; and (5) relative but not absolute hepatropism (29).

4.1 Genomic organization and viral proteins

HBV DNA contains four long open reading frames (ORFs) (Figure 2) (16); the *C* ORF codes for the core protein and secretes hepatitis B e antigen (HBeAg); the *S* ORF codes for the viral envelope and is divided into the pre-*S1* region, pre-*S2* region and *S* gene, which encode the pre-S1, pre-S2 and S proteins (hepatitis B surface antigen, HBsAg); the *P* ORF codes for a protein with several functions in replication (reverse transcriptase, DNA polymerase, RNase H, and primer for DNA synthesis);

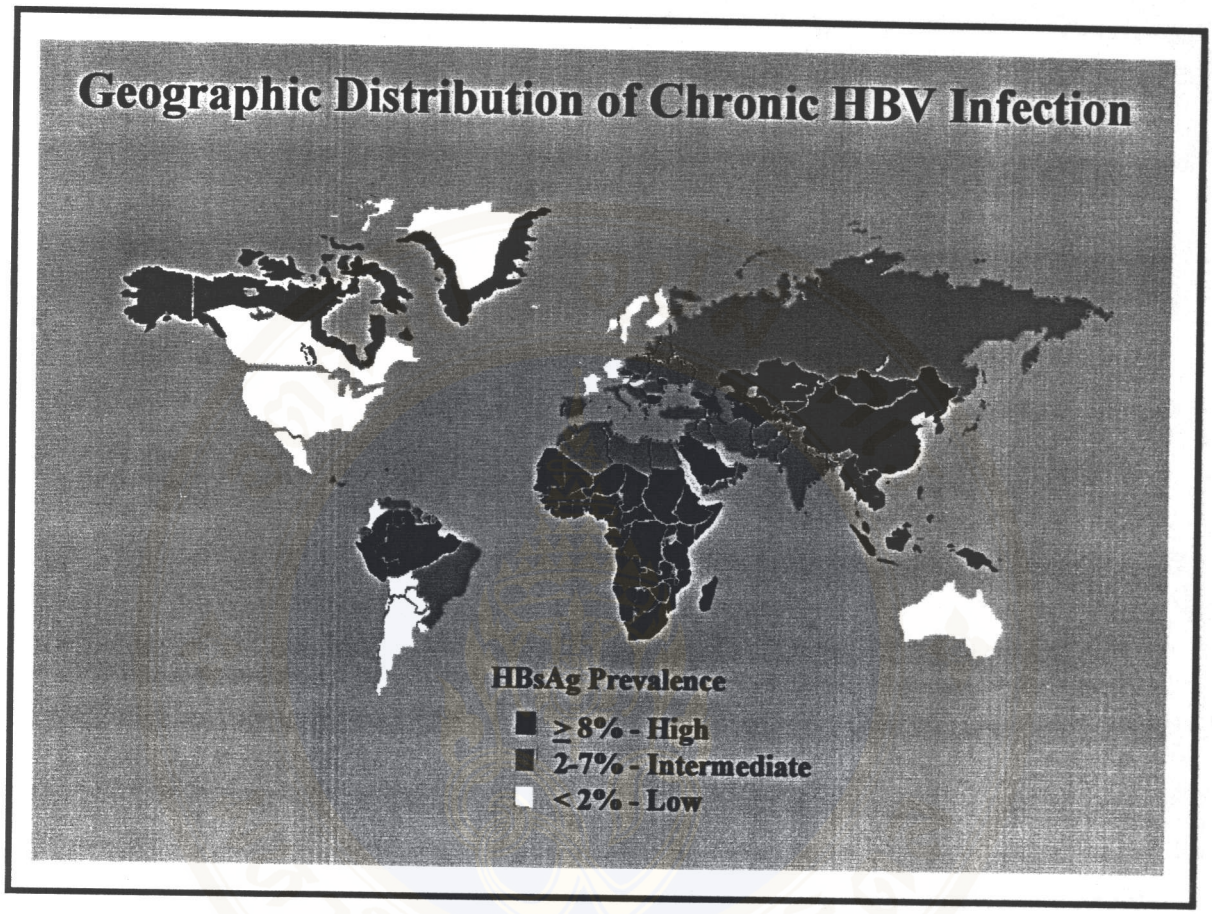


Figure 1. Geographic distribution of chronic HBV infection.
(Obtained from http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep36.gif)

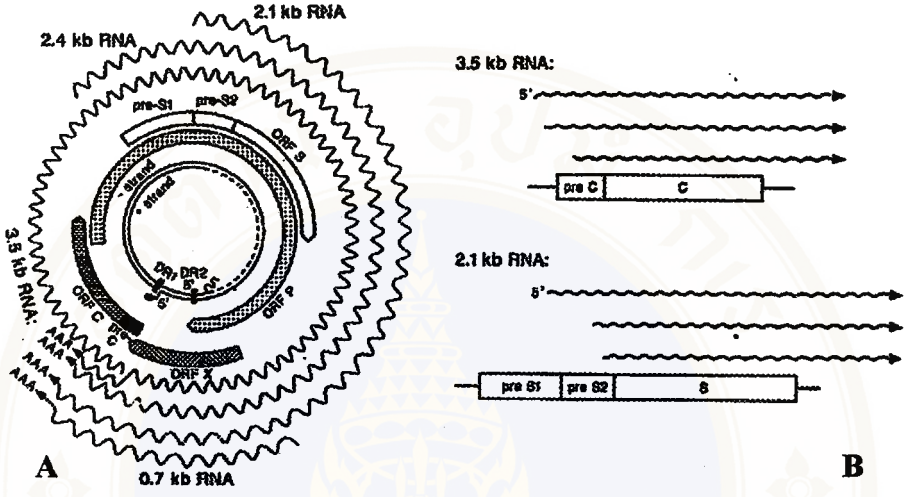


Figure 2. (A) The HBV genome, its transcripts, and its coding regions. Inner circle depicts virion DNA. On this genome,the filled circles indicate terminal proteins on minus strand DNA, and the wavy line denotes terminal RNA on plus strand DNA. DR1, DR2 indicate positions of an 11-element directly repeated twice on the genome; these functions in the initiation of viral synthesis. Boxes denote open reading frames, wavy lines in outer most ring depict the major stable transcript found in HBV infected cell.
(B) The fine structure of the 5' termini of precore/core RNA (top) and the the pre-*S2*/ *S* RNA(bottom), depict above their corresponding coding region [29].

and the *X* ORF codes for the X protein which is a small (17 kDa) regulatory protein that influences the expression of viral and cellular genes (29).

Every nucleotide of the HBV genome is translated, but the principal device employed by nature to enhance coding potential is the use of overlapping reading frames. For example, the entire envelope gene is also used in another frame to encode polymerase sequences.

Another device used in several HBV genes is differential translation initiation at several AUG codons within an ORF. The example is the surface proteins which three related polypeptides are encoded from one large coding region. The ORF of surface proteins contains two upstream and in-frame AUG initiation codon (23, 24). The region upstream of *S* gene is referred to as the pre-*S* region which divided into two subregions by the location of these AUGs (pre-*S1* and pre-*S2*). Initiation of translation in the pre-*S* region give rise to fusion proteins which pre-*S* sequences are merged in-frame to *S* sequences. Thus, the large envelope proteins contain an S protein and variable N-terminal extension.

A similar arrangement also operates within the viral *C* ORF. In this region, two in-frame AUG codons exist; the internal AUG encodes the 21 kDa C protein, and the most 5′ AUG directed another product, the 24 kDa precore protein. The precore region encodes signal sequence that directs the nascent protein into the endoplasmic reticulum where it is removed, and only the remaining polypeptide is then secreted extracellularly. During the secretory pathway, the C-terminal region of peptide chain is removed by host protease to generate a 16 kDa HBeAg fragment (25-28). Thus, the *C* ORF encodes two related proteins that are targeted to different subcellular compartments and presumably serve for different functions in the viral life cycle. HBcAg is used in the construction of the viral nucleocapsid. HBeAg plays no role in viral assembly and its true *in vivo* function is not yet clear.

4.2 The surface protein (HBsAg)

HBV virion (Figure 3) (29) are 40-42 nm, double-shelled particles whose outer lipoprotein envelope contains multiple glycoproteins called the major, middle and the large protein. The major protein, the most abundant protein, is the 24 kDa (226 amino acids long) hepatitis B surface antigen (HBsAg) which is identical to the Au antigen. It is encoded by the *S* gene and has two forms, glycosylated (GP27) and non-glycosylated (P24). GP27 possesses a complex N-linked glycan at position Asn 146. HBsAg is a conformational antigen. A dimer of two major protein molecules linked by disulphide bridges represents the structural unit that bears full HBsAg antigenicity (30). Reduction of disulphide bonds results in dissociation of dimer and a drastic decrease of HBsAg antigenicity.

HBsAg contains a group determinants called 'a' and subtype determinant termed *d,y,w* and *r*. Either of the two pairs of allelic determinants *d/y* and *w/r* is regulated by single amino acid among the 226 residues encoded by *S* gene. Lysine or arginine at position 122 specifies the *d* or *y* determinant, and lysine or arginine at position 160 specifies *w* or *r* determinant, respectively. These amino acid changes, in turn, are attributed by conversion of a single nucleotide, adenine or guanine, at position 365 and 479 in *S* gene; contributing to codons 122 and 160 for lysine (AAA/AAG) or arginine (AGA/AGG) respectively (31). Four main subtypes, *adw*, *adr*, *ayr* and *ayw* are commonly observed and distributed worldwide. The subtypes *adr* and *adw* are found in Thailand with the frequencies of about 80% and 20%, respectively (32). The 'a' determinant is a common epitope of all HBV isolates. This region is conformational epitope and is thought to consist of two loops held by disulfide bridges between cysteines 124 and 137, and cysteines 138 and 147. The second loop is more conserved and confers most of antigenicity of the 'a' determinant (33-37). Antibodies to 'a' determinant confer protection against all subtypes of HBV (38).

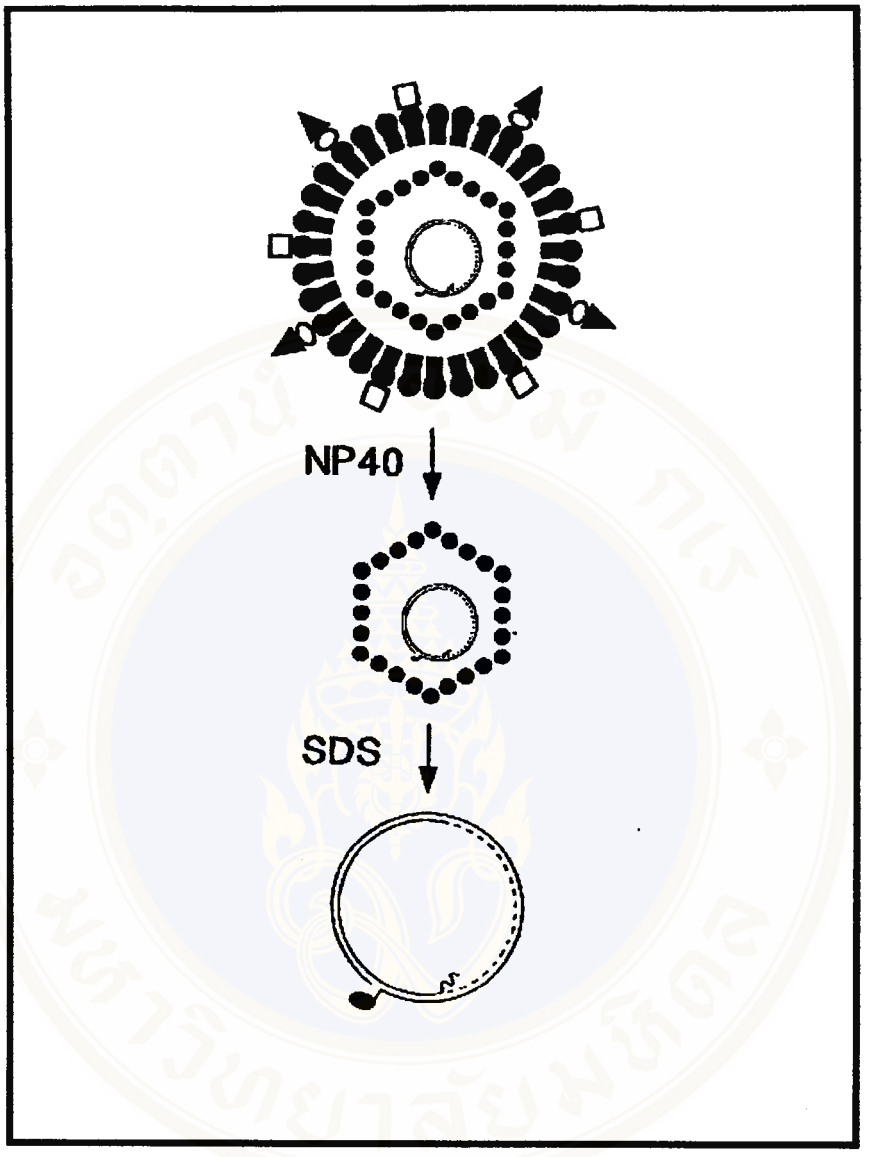


Figure 3. Schematic diagram of HBV virion and subviral particles (top), core (middle), and genome (bottom). The lipoprotein envelope of the virion contains the three surface polypeptides. Envelope removal with the nonionic detergent NP40 liberates the viral core, made up of 180 copies of the C protein in an icosahedrally symmetric capsid. Internal to this is the viral polymerase and genome, which can be extracted from the core by treatment with sodium dodecyl sulfate (SDS)[29].

However, there are some samples of individuals positive for anti-HBs antibodies which are associated with absence of antibodies to 'a' determinant or the emergence of HBV variants (39-41). Moreover, these are some patients who develop HBV reinfection despite hepatitis B immune globulin (HBIG) prophylaxis (42, 43). For examples, Ghany *et al* found amino acid substitution in the 'a' determinant in these patients who were reinfected despite HBIG prophylaxis.

The middle protein is 281 amino acids long and encoded by the pre-*S2* region and *S* gene. It is a glycoprotein and presents in two forms, GP33 and GP36, according to the extent of glycosylation. GP33 contains one glycan and GP36 has two glycans. The 55 amino acids encoded by pre-*S2* region are hydrophilic and contain a domain epitope located at the surface envelope. This epitope is disulphide bond-independent and apparently more immunogenic than the epitopes of the major protein. The pre-*S2* encoded sequence contains a receptor for polymerized human serum albumin (pHSA) (44). Hepatocyte also have a receptor for pHSA and it has been postulated that pHSA mediates the attachment of the HBV to hepatocyte.

The large protein is encoded the pre-*S1* region, pre-*S2* region and *S* gene and is present in a glycosylated (GP42) and a non-glycosylated (P39) forms. This protein is of variable length according to the viral subtype; 389 or 400 amino acids long for the *ay* and *ad* subtype, respectively. GP42 contains one N-linked glycan. At least part of the N-terminal sequence, encoded by the pre-*S1* region, is located at the surface of the envelope. The pre-S1 translation product may also be involved in the attachment of HBV to hepatocytes.

4.3 Core protein

The envelope of the virion can be removed by treatment with nonionic detergents to reveal the inner nucleocapsid. This 27 nm, icosahedral symmetric structure contains 180 units of the viral core (C) protein which also refers to as hepatitis B core antigen (HBcAg). The C protein is a 21 kDa polypeptide composed of two domains, an N-terminal assembly domain that promotes oligomerization into capsids, and a C-terminal whose arginine-rich region implicates in sequence-non specific nucleic acid interaction (45-47). These core proteins are tightly organized and protect the genome within them from exogenous nucleases.

Inside the core, an unusual small DNA molecule with remarkable structure asymmetry is located. The HBV genome is a relaxed circular species which is maintained by the hydrogen bonding of complementary 5′ cohesive end of approximately 250 bp. The minus strand of the viral DNA is unit length (3.2 kb), while its complementary (the plus strand) is less than unit length. The 5′ end of the plus strand is fixed, but its 3′ end can be anywhere along the genome. This means that each molecule of HBV DNA has a single stranded gap which is variable in length from virion.

Another remarkable feature of HBV DNA have been observed. The 5′ end of each viral DNA strand is covalently linked to that viral protein, and plus strand has a covalently attachment to oligoribonucleotide (48). These covalent linkings are important for the initiation of the synthesis of each of two DNA strands. The termini of the minus and plus strands map to the short (11-12 nt) sequences regions that are directly repeated twice on the viral DNA circle; these direct repeats are known as DR1 and DR2. They play an important role in directing the initiation of viral DNA synthesis (49).

4.4 Polymerase

Within the core is also a HBV DNA polymerase, which has ability to fill in single strand region of viral DNA by using the 3′ end of the plus strand DNA as a primer and the minus strand as a template. The product of this endogenous polymerase reaction is a fully duplex relaxed circular form of the viral DNA. This viral polymerase is a histidine-rich protein which is encoded by polymerase *P* gene, and is also the linkage protein at the minus strand termini. In HBV replication process, the P protein is required for the synthesis of both strands of viral DNA that involves the reverse transcription of an RNA intermediate. Thus, the P protein is actually a reverse transcriptase (RT) which is strongly supported by the subsequent recognition of sequence homologies with retroviral reverse transcriptases.

4.5 X Protein

X protein which comprises 154 amino acids, may plays a major role in hepatocarcinogenesis. The *X* gene exhibits a trans-activation function through X-responsive elements for viral and cellular gene, such as SV40, c-Fos, c-Jun, and RNA polymerase II gene. However, X protein analyzed so far has no direct DNA-binding activity. Therefore, its trans-activating and/or oncogenic function is probably manifested through protein-protein interaction with a number of cellular proteins.

5. HBV Genotype

HBV can be classified in to six genotypes, A to F (50, 51). The global molecular epidemiology of HBV has been revealed by comparing the *S* gene from a large number of HBV strains of diverse geographical origin. The genotypes have a characteristic geographical distribution and are in general linked to HBsAg subtypes (52). Thus strains belonging to genotype A are mainly found in Northern Europe, USA and sub-Saharan Africa. Genotype B and C are found in Southeast Asia and the Far East including Thailand. Genotype D is found worldwide, but is the dominating genotype of the Mediterranean area, the Near and Middle East and South Asia. Strains belonging to genotype E have so far only been reported from sub-Saharan Africa, and genotype F, corresponds to *adw4* subtype, represents the HBV strains of the Ameridian populations of the Americas.

6. Viral Replication

The main features of the HBV replication cycle are summarized in Figure 4 (16), the principal feature of which is that the DNA genome is replicated by reverse transcription of an RNA intermediate. The HBV replication cycle is believed to consist of the following steps. First, after penetration of the viral nucleocapsid into the hepatocyte and transport to the nucleus, the circular double stranded DNA is repaired to a supercoiled DNA forms (the covalently closely circular DNA, cccDNA). Second, the minus strand is transcribed by the cellular RNA polymerase II to the longer (precore) and shorter (pregenomic) 3.5 kb RNA, and pregenomic RNA are synthesized from each genome. Third, the pregenomic RNA is encapsidated with DNA polymerase and core protein. The minus strand DNA is then synthesized by reverse transcription of pregenomic RNA. Initiation of reverse transcription takes place at DR1 and the DNA-linked protein covalently bound to the 5´ end of the minus strand probably acts as a primer for minus strand synthesis.

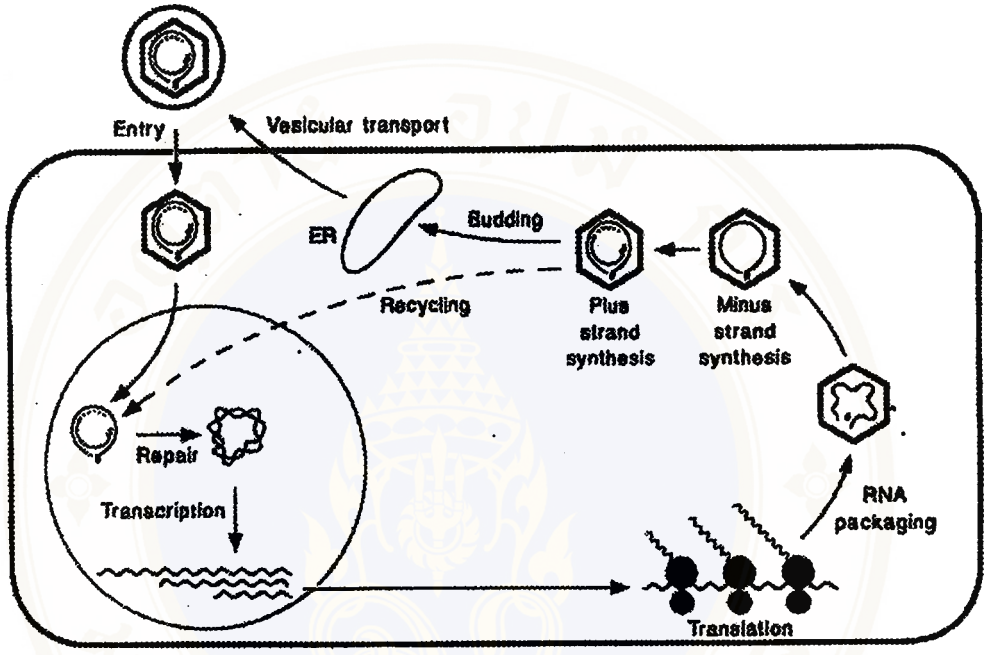


Figure 4. The HBV replication cycle. ER, endoplasmic recticulum [29].

During DNA synthesis, the pregenome is degraded by an RNase H-like activity. So that the product of reverse transcription is a full-length minus DNA strand. Fourth, a small RNA fragment of about 20 bp, probably derived from 5´end of the pregenome, act as a primer which is used to prime the synthesis of the plus strand at DR2. Complete synthesis of this strand is not necessary for coating of the nucleocapsids and export of virus particles.

The transcription of HBV DNA produces the longer and shorter 3.5 kb RNA which were controlled by core promoter. The core promoter region of HBV has two components: the basal core promoter region (BCP) between at nucleotides 1744 to 1851 and the core upstream regulatory sequence (CURS) between at nucleotides 1636 to 1743. BCP controls the production of both pregenomic RNA and precore RNA. The pregenomic RNA has multiple functions: it codes for the core protein, which is the major capsid protein serves as the template RNA, which is packaged into viral cores, and also codes for the DNA polymerase, which reverse transcribes the pregenomic RNA into partially double-stranded DNA genome. The precore RNA codes for the precore protein which is the precursor of HBeAg.

In the course of HBV infection, the presence of HBeAg is generally correlated with active viral replication and often liver disease. On the other hand, clearance of HBeAg and subsequent rise of the anti-HBe antibody indicate termination of HBV replication with or without integration of HBV DNA, and remission of liver disease (1). However, some patients, particularly in Mediterranean countries and the Far East, have persist viremia and severe active liver disease even after clearance of HBeAg.

For example, 14/18 (78%) of HBsAg-positive/anti-HBe-positive patients with chronic liver disease were positive for nuclear HBcAg, serum HBV DNA, or both of these markers of HBV replication (2, 3). In these individuals, active chronic liver disease appears to be related to continued replication and secretion of HBV. Tong *et al* cloned and sequenced HBV DNA from the serum of three anti-HBe-positive patients. The DNA sequencing revealed an inactive precore region in all 11 HBV clones, either as one point mutations in the 3' terminus generating an in-frame TAG (1895-1897) stop codon, or a 1 nucleotide insertion in the 5' terminus (T,1828) resulting in frameshift mutation. Furthermore, the complete 3182 nucleotide sequence of a clone was determined and no significant mutation was found in the remainder of the genome. They concluded that chronic hepatitis patients positive for anti-HBe antibodies are associated with HBV variants containing an inactive precore region and hence cannot synthesize precore region-derived HBeAg (4).

Independently, Carman *et al* (5) found that some patients with chronic hepatitis B virus (HBV) infection are HBeAg-negative, but have circulating HBV particles, and often have especially severe chronic hepatitis. In 7 out of 8 HBeAg-negative patients, two mutations (guanosine to adenosine) were found in the terminal two codons of the precore region, giving the sequence 1895TAGGACATG1903. The remaining patients had the first mutation only. The sequence 1895TGGGGCATG1903 was found in 4 of 5 of the HBeAg-positive patients. The first mutation (TAG) results in a translational stop codon that is predicted to result in failure to produce HBeAg. The rest of the precore region in the HBeAg-negative patients was otherwise homologous to that of the HBeAg-positive patients and to known sequences. The predominant mutation is G to A at nucleotide 1896 (G1896A), leading to a stop codon (TGG to TAG) at codon 28. The stop codon mutation in the precore region of HBV prevent HBeAg secretion but still permit HBV replication and HBcAg production.

These mutations are also detected in patients with fulminant hepatitis B. Eighteen patients with fulminant hepatitis B were studied. Of the 15 cases from whom serum viral DNA could be sequenced, the variant was found in the admission sera of 8 of 9 HBeAg-negative patients but in none of 6 HBeAg-positive patients who had fulminant hepatitis B. Patients harboring the variant progressed more rapidly into hepatic encephalopathy, but those infected with the variant strain alone had a greater likelihood of survival than those infected with the normal strain or a mixture. The mutant strain may emerge spontaneously during fulminant hepatitis as occurs in chronic hepatitis B infection during seroconversion from HBeAg to antibody (6, 8).

Carman *et al* (7) classified the prevalence and variety of the mutant from locations where particular HBV subtypes predominate, and the predominant strain correlates with the serologic picture in chronic hepatitis patients with fluctuating HBeAg status. Four mutations in the precore region were found. M1 involved a nucleotide substitution from C to T at nucleotide 1856 (C1856T) leading to an amino acid change from proline to serine in codon 15. M2 consisted of a G to A mutation at nucleotide 1896 (G1896A) creating a stop codon at codon 28. M3 was a G to A mutation at nucleotide 1898 (G1898A) giving rise to an amino acid change from glycine to serine in codon 29. M4 was a G to A mutation at nucleotide 1899 (G1899A), after the stop codon and thus untranslational. They found that an association between isolates containing M1 or M2 and severe disease in the anti-HBe-positive phase of chronic hepatitis, and it is not known whether M1 is found in patients with fulminant hepatitis, as shown for M2. In contrast, some patients with normal ALT levels and very low level viremia can be infected with either mutants, as found for M2 in Japanese patients.

The prevalence of precore HBV mutants in HBeAg-positive and HBeAg-negative asymptomatic carriers were determined (53). Wild type sequence was more often found in HBeAg-positive than in HBeAg-negative family member (76% vs. 45.5%; p=0.02), and M1 was equally distributed among HBeAg-negative family members and HBeAg-positive family members. M2 was detected more frequently in HBeAg-negative family members (45.5% vs. 4.5% in HBeAg-positive family members; $p < 0.0001$). However, the level of ALT were normal in most of among asymtomatic carriers. These mutants are not necessarily associated with active liver disease. Other variations have been found including the loss of precore initiation codon, nucleotide insertions and deletions resulting in frameshift mutation (9), and T to C substitution at position 1815 (T1815C) that eliminates the start codon of the precore transcription (10).

HBV mutants unable to produce HBeAg often become the dominant viral quasispicies in the viral population present in the infected individual. The mechanism for their increased fitness may involve selection via the immune response or by enhancement of viral replication rates. The mutations associated with the loss of HBeAg production in patients frequently include stop codons and frameshift mutations within the precore region. Analyses of the mutations occurring in the BCP of Japanese patients with fulminant hepatitis revealed a correlation between HBeAg-negative phenotype and frequently observed double mutation of A to T at nucleotide 1762 (A1762T) and G to A at nucleotide 1764 (G1764A). The implication of this observation was that this double mutation in the BCP could also prevent HBeAg expression (11).

However, there were studies that showed the enchanced replication due to different mechanisms. One study showed an increased pregenomic RNA level (54), but the other study showed only enchanced encapsidation but no increased concentration of this RNA (55). In contrast to this finding (T1762/A1764 variants), Laskus *et al* examined the BCP in HBV infected individual from the United States and found that while this double mutation was frequently observed, it was not correlated with HBeAg status of patients (12). Interestingly, by studying another cohort of Japanese patients, Takahashi *et al* suggested that this double mutation suppressed but did not abolish HBeAg expression and hence have termed this mutation an e-suppressive mutation. Thus there is a discrepancy concerning the effect of this BCP double mutations the production of HBeAg (13).

Although, the correlation between HBeAg expression and mutation of the core promoter region has been demonstrated but the mechanism is unclear.

7. Laboratory Diagnosis

The laboratory diagnosis of acute hepatitis is made by biochemical test of liver function and immunological assay. The initial laboratory evaluation of a jaundice patient should include the following tests: total and direct bilirubin, ALT, AST, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase, albumin and globulin and complete blood count (CBC). ALT, AST, ALP are sensitive indicators of hepatocellular damage. These tests are useful for evaluating patients with chronic hepatitis and also with acute hepatitis. Hypoalbuminemia, hypergammaglobulinnemia, elevation of the prothrombin time and hyperbilirubinemia are indicators of more serious chronic liver disease and presumably cirrhosis, often confirmed by a percutaneous or laparoscopic liver biopsy. Immunological assays are essential for confirming viral hepatitis B infection, and the terms used in identifying the various serologic markers are summarized in Table 1.

Table 1. Interpretation of HBV serologic markers in patients with hepatitis (16).

Assay result			
HBsAg	Anti-HBs	Anti-HBc	Interpretation
Positive	Negative	Negative	Early acute HBV infection.
Positive	(±)	Positive	HBV infection, either acute or chronic. Differentiate with IgM anti-HBc. Determine level of replicative activity (infectivity) with HBeAg or HBV DNA.
Negative	Positive	Positive	Indicates previous HBV infection and immunity to hepatitis B.
Negative	Negative	Positive	Possibilities include; HBV infection in remote past; 'low-level' HBV carrier; 'window' between disappearance of HBsAg and appearance of anti-HBs; or false-positive or nonspecific reaction. Investigate with IgM anti-HBc, and/or challenge with HBsAg vaccine. When present, anti-HBe helps validate the anti-HBc reactivity.
Negative	Negative	Negative	Another infectious agent, toxin injury to liver, disorder of immunity, hereditary disease of the liver, or disease of biliary tract.
Negative	Positive	Negative	Vaccine-type response.

Serologic assays for the diagnosis of acute and chronic HBV infection using HBsAg and HBeAg are both sensitive and specific. HBsAg, HBeAg, anti-HBc and anti-HBe are detected by standardized enzyme immunoassay (EIA). The detection of HBsAg indicates active HBV infection, and the detection of HBeAg indicates active viral replication and increased infectivity. Characteristic serologic changes develop in relationship to clinical symptoms and biochemical abnormalities in acute resolving infection (Figure 5A) and acute followed by chronic infection (Figure 5B) (56).

Another tests have been used to examine pathological specimens for HBV infection such as immunofluorescence, *in situ* hybridization, immunohistochemistry and thin-section electron microscope. These tests provide important insights into the relationship between HBV DNA replication and gene expression at the single-cell level.

For example, during active replication of the virus, HBsAg is localized to the cytoplasm, whereas HBcAg can be demonstrated in the nucleus and/or the cytoplasm within the hepatocyte. Nucleic acid hybridization procedures and the PCR assay are used to detect HBV DNA in serum and tissue. In this regard, HBV DNA studies have made important contributions to the evaluation of antiviral therapy. Filter or liquid-phase hybridization assays can detect quantities of HBV DNA sequences from 0.1 to 1.0 pg (28,000 to 280,000 genomic equivalents). A branched DNA signal amplification assay has a cutoff level of about 700,000 genomic equivalents per milliliter. In contrast, the PCR assay can detect as few as 250 HBV DNA molecules per milliliter, which is over 100-1,000 times more sensitive than the hybridization techniques. Moreover, this assay can detect virus genome 2 to 3 weeks before the appearance of HBsAg in experimentally infected chimpanzees and is still detectable for 1 to 3 weeks after anti-HBs seroconversion.

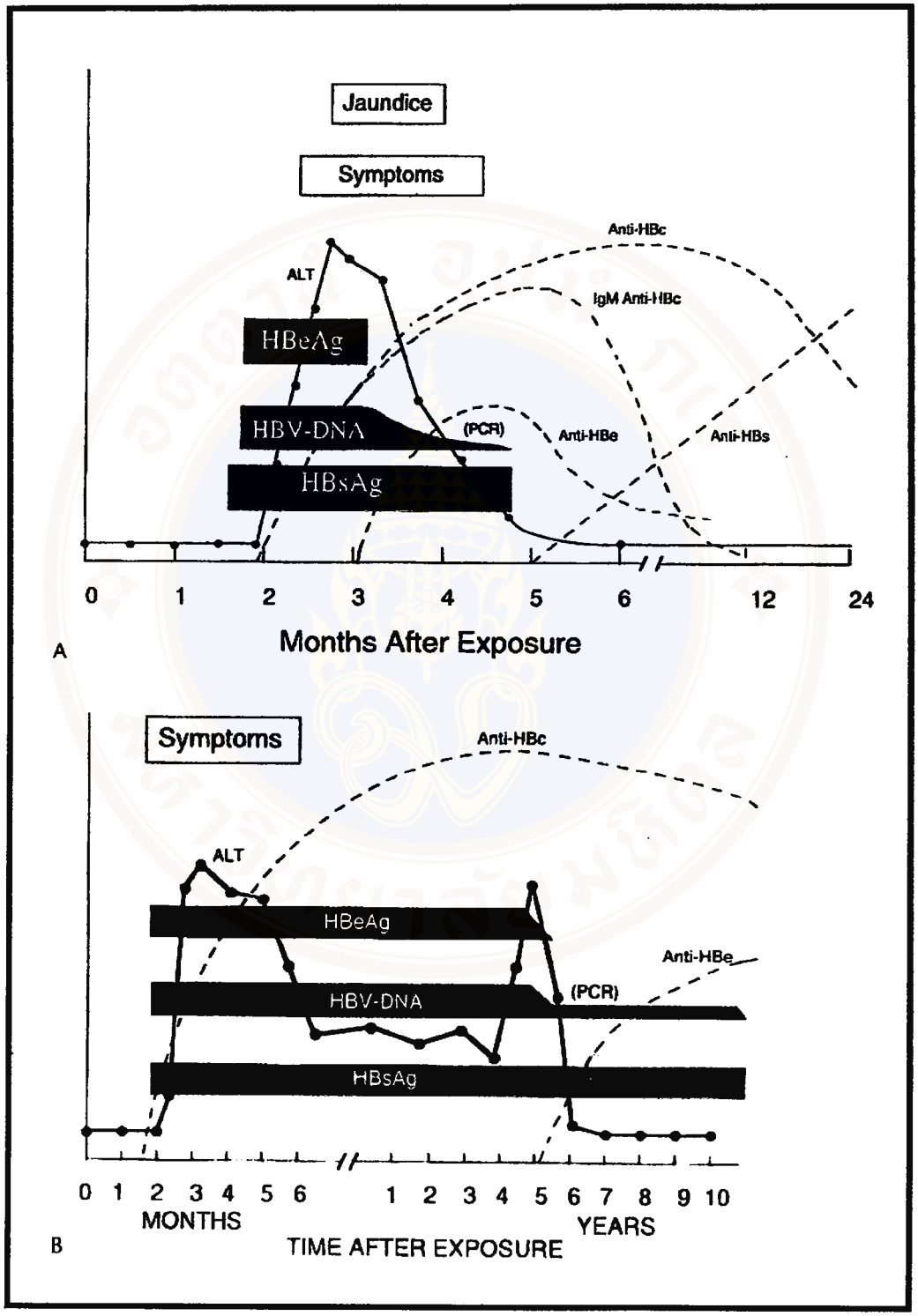


Figure 5. Sequences of events after acute infection. (A) Acute HBV infection with resolution (for explanation of these changes see text).
(B) Acute HBV infection progressing to chronicity (for explanation of these changes see text) [29].

CHAPTER IV

MATERIALS AND METHODS

1. Subjects

1.1 Patient samples

Serum samples for the study of the core promoter region, precore region and the *S* gene were obtained from 105 chronic hepatitis B patients (116 samples) who were tested positive for HBsAg. These patients consecutively attended the Hepatitis Clinic, Faculty of Medicine, Siriraj Hospital, during 1994-1998.

1.2 Normal samples

1.2.1 Serum samples for the detection of anti-HBs antibody by ELISA were obtained from 41 medical students who were immunized with HBV vaccine. The samples were collected before and after vaccination (pre-vaccination samples and post-vaccination samples) by the Division of Virology, Department of Microbiology, Faculty of Medicine, Siriraj Hospital. Positive control serum was obtained from a person who has over 1,000 mIU/ml of anti-HBs antibody, and the negative control serum was obtained from a person who has no anti-HBs. All these samples were tested for anti-HBs antibodies by a commercial ELISA kit (AUSAB EIA, Abbott laboratories, Diagnostic Division, Abbott Park, IL, USA) which showed negative result in all 41 pre-vaccination samples and positive result in all 41 post-vaccination samples.

1.2.2 Serum samples were obtained from 47 healthy persons who had no anti-HBs antibodies as determined by passive hemagglutination test (PHA, Hebsgencell, International Reagents Corporation, Japan). These samples were collected by the Hepatitis Section, National Institute of Health, Department of Medical Science, Ministry of Public Health, during 1992-1997.

2. Oligonucleotide Primers

Oligonucleotide primers, specific for *S* gene and *C* gene sequences (HBs1, HBs2, B1778E and B2017), were designed by Dr. Sirirurg Songsivilai, Department of Immunology, Faculty of Medicine, Siriraj Hospital. The primers of the core promoter region and precore region (eP 1-1 and eP 1-2) were used as designed by Takahashi *et al.*, 1995 (13). The details of primers are described in Table 2.

Table 2. Nucleotide sequences and position of primers

Primer	Nucleotide Sequence (5'->3')	Positions [a]	Polarity [b]
B1778E	gACgAATTCCATTgACCCgTATAAAgAATT	1897-1926	+
B2017	ATgggATCCCTggATgCTgggTCTTCCAAA	2125-2154	-
HBs1	ACCCATATCgTCAATCTYCTCg	109-130	+
HBs2	CCATgAAgTTAAgggARTACRC	859-880	-
eP 1-1	gCATggAgACCACCgTgAAC	1606-1625	+
eP 1-2	ggAAAgAAgTCAgAAggCAA	1955-1974	-

[a] The position 1 was given with respect to the *Eco*RI site

[b] +, sense; -, antisense polarity

3. Isolation of DNA from Serum

DNA was isolated from serum samples by heat treatment method (57). One hundred microliters of serum was incubated at 100°C for 5 minutes in Dri-Bath (Type 17600, Thermolyne, Iowa, USA) and then centrifuged at 13,000 rpm (Sorvall, model RMC 14, Delaware, USA) for 15 minutes at 25°C. The supernatant was collected and kept at -70°C. Serum samples with or without HBV-DNA were included in parallel in each isolation as positive and negative controls, respectively.

4. DNA Amplification

4.1 *S* gene and *C* gene regions

PCR amplification was carried out in a 100 µl mixture containing 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 200 µM of each of dNTPs (Promaga, Wisconsin, USA), 1.5 mM $MgCl_2$, 50 pmole of each sense and antisense primers, and 2.5 units of T*aq* DNA polymerase (Perkin Elmer, New Jersey, USA) and 20 µl of DNA sample. Steriled deionized water was added to bring the final volume to 100 µl. The mixture was subjected to 40 PCR cycles, each consisted of 1 minute at 94°C for denaturation, 1 minute at 42°C for annealing and 1 minute at 72°C for extension. The final extension step was at 72°C for 10 minutes in an thermalcycler (GeneAmp PCR system 2400, Perkin Elmer, USA). Amplification of HBV DNA of *S* gene was performed using primers HBs1 and HBs2, giving the PCR-amplified product size was 772 base pairs (bp). The size of PCR-amplified product of the *C* gene using primers B1778E and B2017, was 257 bp.

4.2 The core promoter region and precore region

PCR amplification was carried out in a 50 µl mixture containing 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 200 µM of each of dNTPs (Promaga), 1.5 mM $MgCl_2$, 50 pmole of each sense and antisense primers, and 1.25 units of T*aq* DNA polymerase (Perkin Elmer) and 10 µl DNA sample. Steriled deionized water was added to bring the final volume to 50 ml. The mixture was subjected to 40 PCR cycles, each consisted of 1 minute at 94°C for denaturation, 1 minute at 50°C for annealing and 1 minute at 72°C for extension. The final extension step was at 72°C for 10 minutes in an thermalcycler (GeneAmp PCR system 2400). Amplification of HBV for the core promoter region and precore region was performed using primers eP 1-1 and eP 1-2, and the PCR-amplified product size was 369 bp.

5. Detection of PCR-amplified Product by Agarose Gel Electrophoresis

5.1 Agarose gel preparation

Agarose gel was prepared at a concentration of 2% (w/v) in 1xTBE buffer (see appendix), melt in microwave oven until completely dissolved and then poured into the gel box (model Sub-cell GT mini, BioRad, California, USA) with an appropriate comb.

5.2 Preparation of PCR-amplified product for loading and gel electrophoresis

Five microliters of PCR-amplified product was thoroughly mixed with 6x loading buffer (see appendix). The PCR-amplified product was loaded into the submarine 2% agarose gel, and electrophoresis was carried out at constant volt of 110 volts for 45 minutes or until the bromophenal blue dye reached about 1 cm from the lower edge of the gel, then the electrophoresis was stopped.

After electrophoresis, the agarose gel was stained with ethdium bromide by soaking the gel in a solution containing 10 µg/ml of ethidium bromide, and then visualized under ultraviolet (UV) light (Fotodyene, Wisconsin, USA). The gel was photographed using Bio-Rad's Image Analysis System (model Gel Doc 1000, BioRad, California, USA).

6. Preparation of DNA Template for Nucleotide Sequencing Reaction

6.1 Low melting point (LMP) agarose gel electrophoresis and purification of DNA for *S* gene

The PCR-amplified product was mixed with 6x loading buffer and then loaded into the 1.5% (w/v) LMP agarose gel (Gibco BRL, Maryland, USA) in 1xTBE buffer. Electrophoresis was carried out at the constant volt of 75 volts until the bromophenal blue dye reached about 1 cm from the lower edge of the gel, then the electrophoresis was stopped. The gel was stained and viewed as previously described.

DNA band of the expected size was cut from gel by a clean blade and placed into a new 1.5 ml microcentrifuge tube. The tube containing gel slice was incubated in a Dri-Bath at 70°C until the gel was completely melted. The DNA was purified using Wizard 373 DNA Purification System (Promega). One microliter of well-mixed resin was added into the tube which contained completely melted gel and mixed by vortex for 1 minute. The mixture was stood at room temperature for 5 minutes and then transferred to a 2.5 ml syringe barrel which was contacted to a minicolumn provided with the kit. Plugger was applied to the barrel and pushed slowly to let the mixture pass through the minicolumn.

The minicolumn was washed with 2 ml of washer solution (80% isopropanol), spinned at 10,000 rpm (Tomy refrigerated microcentrifuge model MRX150, Tokyo, Japan) for 10 seconds to dry off minicolumn. The minicolumn was left at room temperature for 15 minutes to let any residual of washer solution to evaporate. DNA was eluted from the minicolumn by adding 20 µl of distilled water and then the minicolumn was spinned for 1 minute to collect the DNA elute. This DNA was kept at -20°C and used as template for nucleotide sequencing reaction.

6.2 Glassmilk-based purification for the core promoter region and precore region

The PCR-amplified product was purified by glassmilk-based DNA purification using Geneclean II kit (Bio 101 Inc., California, USA). The PCR-amplified product was taken from the reaction tubes to a new 1.5 ml microcentrifuge tube. Three volumes of sodium iodide (NaI) solution was added to the DNA solution, and 1/10 volume of TBE modifier or 1/200 volume of 10 % acetic acid were added in the tube followed by 5 µl of glassmilk. TBE modifier or 10 % acetic acid were used to lower the pH of the NaI so that smaller DNA molecules of 200-500 bp could bind with a higher efficiency to glassmilk.

The mixture was mixed thoroughly by vortexing and then kept on ice for 5 minutes to allow DNA to bind to glassmilk. DNA-glassmilk was collected from the solution by spinning the tube at 10,000 rpm for 5 seconds, and the supernatant was discarded. DNA-glassmilk pellet was washed three times with 300 µl of precooled washing buffer and the DNA was eluted from glassmilk by adding 20 µl of distilled water into the pellet and incubated at 50°C for 5 minutes. After spinning the tube for 1 minute at 10,000 rpm, DNA solution was pipetted out from the glassmilk pellet and kept at -20°C.

7. Automated DNA Sequencing Using Dye Terminator System

7.1 Nucleotide sequencing reaction of *S* gene

For automated DNA sequencing, PCR-amplified product for *S* gene was prepared using Wizard 373 DNA Purification System. The purified PCR product was used for the sequencing reaction using Prism Ready Reaction DyeDeoxy Terminator FS Cycle Sequencing Kit (Applied Biosystem, Inc., California, USA) according to manufacturer's instruction. The DNA template was mixed with 8 µl of Prism Terminator Mix, 3.2 pmole of primer and distilled water was added to bring the final volume to 20 µl. The sequencing reaction was subjected to 25 PCR cycles, each consisted of 96°C for 30 seconds , 50°C for 15 seconds and 60°C for 4 minutes in an thermalcycler (GeneAmp PCR system 2400).

The sequencing reaction mixture was then purified using phenol-chloroform extraction method. Briefly, the sequencing reaction was added 80 µl of deionized water, bring to final volume 100 µl and then was extracted twice with an equal volume of phenol:chloroform:deionized water (68:14:18). The sequencing fragments were precipitated by adding 15 µl of 2 M sodium acetate (pH 4.5) and 300 µl of absolute ethanol, vortexed briefly, and then spinned at 10,000 rpm 4°C for 15 minutes. The sequencing pellet was washed once with 500 µl of 70% ethanol and then dried for 30 minutes at room temperature in the dark.

7.2 Nucleotide sequencing reaction of the core promoter region and precore region

PCR-amplified product for the core promoter region and precore region was prepared using Geneclean II kit. The purified PCR product was used for the sequencing reaction using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem). The DNA template was mixed with 4 µl of Terminator Ready Reaction Mix, 3.2 pmole of primer and distilled water was added to bring the final volume to 20 µl. The sequencing reaction was subjected to 25 PCR cycles, each consisted of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes in an thermalcycler (GeneAmp PCR system 2400).

The sequencing reaction was purified using simplified ethanol precipitation. The sequencing reaction was added with 16 µl of deionized and 64 µl of 95% ethanol, vortexed briefly, and stood at room temperature in the dark for 15 minutes. The precipitate of sequencing reaction was centrifuged at 12,000 rpm at 4°C for 20 minutes, and the pellet was washed with 250 µl of 70 % ethanol. The suspension was re-centrifuged at 12,000 rpm at 4°C for 10 minutes, and the pellet was dried for 1 minute at 90°C in a Dri-Bath. The pellet could be kept at -20°C for several months whereas the sequencing reaction could be kept as solution at -20°C for 1 week.

8. Preparation of Injected Sample and Capillary Electrophoresis

The pellet of each sample was resuspended by 25 µl of template suspension reagent (TSR), vortexed and spinned at 12,000 rpm in 4°C for 1 second. The suspension was heated at 95°C for 2 minutes in a Dri-Bath, and soaked in ice box immediately for 1 minute.

The suspension was mixed by vortex, spinned at 12,000 rpm in 4°C for 1 second, transferred to sequencing tube and loaded on ABI 310 Genetic Analyzer (PE-Applied Biosystems). The DNA was electrically injected into the capillary for 30 seconds at 2.5 kV and subjected to 90 minutes of capillary electrophoresis at 12.2 kV at 50°C in POP-6 polymer. Raw electrophoregram was collected after laser excitation using the Data Collection software (PE-Applied Biosystems). The sequence data was automatically called using the Sequence Analysis software which analyzed the electrophoregram pattern in comparison with the dye matrix file.

9. DNA Sequence Analysis

The nucleotide sequences were aligned and analyzed with other nucleotide sequences deposited in GenBank database (National Center for Biotectechnology Information, Bethesda, USA) using BLAST and MacVector software version 4.5.3 (Oxford Biomolecular Group, Oxford, UK).

For phylogenetic analysis, evolutionary distances were estimated by DNADIST (for nucleotide sequences) software, the distances was clustered into phylogenetic groupings by NEIGHBOR software and phylogenetic trees were drawn by DRAWTREE (unrooted tree) software. Programs for phylogenetic analysis were available in Phylogenetic Inference Package (PHYLIP) 3.5.7.2 written by Dr. Joseph Felsenstein, Department of Genetics, University of Washington, Seatle, Washington, USA.

10. Synthetic Peptide and Recombinant HBsAg

Synthetic peptide containing amino acid residues 121-147 (CKTCTTPAQGTSMFPSCCCTKPSDGNC) of HBsAg was designed based on the sequences of the *S* gene of 10 HBV isolates from Thai patients. The peptide was synthesized using Fmoc solid-phase method. This peptide was ordered and purchased from Bio-Synthesis, Inc., Taxas, USA. The yeast-derived HBsAg was a gift from University of Colorado, USA.

11. Optimization of Conditions of ELISA for Anti-HBV antibody

Indirect ELISA was used in this study. In order to establish the assay, the variation of conditions were carried out to determine the optimal condition of the reagents in this assay.

11.1 Peptide

11.1.1 Determination of microtiter plate types

Various types of 96 well microtiter plate such as MaxiSorp, PolySorp (Nunc-Immuno module, Nunc A/S, Kamstrupvej, Roskilde, Danmark) and Immulon II (Dynatech Laboratories, Inc., Verginia, USA), were coated with 1 µg/ml of peptide in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Between each step, the plates were washed four times in PBS with 0.05% Tween 20 by using the automated washing machine (GLW 1000 Multi-reagents Wash, Genelabs Diagnostic, Singapore, Singapore). The plates were blocked for 2 hours at room temperature (RT) with 200 µl/well of PBS containing 2% skimmed milk (blocking buffer) to reduce nonspecific binding. Control sera (anti-HBs-positive and anti-HBs-negative samples) were diluted 1:10 in blocking buffer, added into the well (100 µl/well) and then incubated for 1 hour at 37°C. The plates were incubated with 100 µl/well of peroxidase conjugated anti-human IgG (Kirbegaar & Perry Laboratory Inc., Maryland, USA) which was diluted 1:20,000, for 1 hour at 37°C. The color was developed by incubation for 30 minutes at room temperature with 10 µg of 3,3´5,5´ tetramethyl benzidine dihydrochloride (TMB) in citrate-phosphate buffer pH 5.0 containing hydrogen peroxide (H_2O_2) (100 µl/well), and the reaction was stopped by the addition of 50 µl/well of 2N H_2SO_4. The absorbance was read at 450 and 620 nm using an automatic microplate reader (Model GLR 1000, Genelabs Diagnostic, Singapore, Singapore).

11.1.2 Determination of the concentration of peptide

Various concentrations of peptide, at concentration 1.25, 2.5, 5.0 and 10 µg/ml in 0.05 M carbonate buffer pH 9.6 (100 µl/well) were used for coating the PolySorp plates, overnight at 4°C. The assay was then performed as 11.1.1.

11.1.3 Determination of the types of blocking buffer

The PolySorp plate was coated with 1 µg/ml of peptide in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Subsequent steps were performed as in 11.1.1. Several types of blocking buffers were used, including 2% skimmed milk, 1% human serum albumin (HSA) and commercial blocking reagent, to reduce nonspecific binding.

11.1.4 Determination for dilution of sample

The PolySorp plate was coated with 1 µg/ml of peptide in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Subsequent steps were performed as in 11.1.1. The positive control and negative control sera were used at the dilutions of 1:10, 1:100 and 1:1000 in blocking buffer.

11.1.5 Determination for concentration of conjugate

Assay was performed as in 11.1.1. The concentrations of the conjugate was various form 1:10,000 and 1:20,000, to optimize the concentration of the conjugate.

11.1.6 Optimization of the ELISA using biotin and streptavidin system

The assay was performed as in 11.1.1. The conjugate system used was changed to the biotin-streptavidin system. The plate was incubated with biotin conjugated anti-human IgG which was diluted 1:10,000 (100 µl/well), for 1 hour at 37°C. Streptavidin-HRP was diluted 1:20,000, added to the well and then incubated for 30 minutes at RT.

The color was developed by incubation for 30 minutes at room temperature with 10 µg of 3, 3′, 5, 5′, tetramethyl benzidine dihydrochloride (TMB) in citrate-phosphate buffer pH 5.0 containing hydrogen peroxide (H_2O_2) (100 µl/well), and the reaction was stopped by addition of 50 µl/well of 2N H_2SO_4. The absorbance was read at 450 and 620 nm using an automatic microplate reader.

11.2 Recombinant HBsAg

11.2.1 Determination of microtiter plate types

Three types of 96 well microtiter plate, MaxiSorp, PolySorp and Immulon II, were coated 0.5 µg/ml of recombinant HBsAg in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Between each step, the plates were washed four times in PBS with 0.05% Tween 20 by using the automated washing machine. The plates were blocked for 1 hours at 37°C with 200 µl/well of PBS containing 2% skimmed milk (blocking buffer) to reduce nonspecific binding. Control sera (anti-HBs-positive and anti-HBs-negative samples) were diluted 1:10 in blocking buffer (100 µl/well) and add to the plates and then incubated for 30 minutes at 37°C. The plates were incubated with peroxidase conjugated anti-human IgG which was diluted 1:20,000 (100 µl/well), for 30 minutes at 37°C. The plates were developed by incubation for 30 minutes at room temperature with 10 µg of 3, 3′, 5, 5′, tetramethyl benzidine dihydrochloride (TMB) in citrate-phosphate buffer pH 5.0 containing hydrogen peroxide (H_2O_2) (100 µl/well), and the reaction was stopped by addition of 50 µl/well of 2N H_2SO_4. The absorbance was read at 450 and 620 nm using an automatic microplate reader.

11.2.2 Determination for dilution of serum

The PolySorp plate was coated with 0.5 µg/ml of recombinant protein in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Subsequent steps were performed as 11.2.1. The positive control and negative control sera were used at the dilutions of 1:10 and 1:100.

11.2.3 Determination for concentration of conjugate

Assay was performed as in 11.2.1. The concentrations of the conjugate was various form 1:10,000 and 1:20,000, to optimize the concentration of the conjugate.

11.2.4 Determination of the types of blocking buffer

The PolySorp plate was coated with 0.5 µg/ml of recombinant protein in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Subsequent steps were performed as 11.2.1. Several types of blocking buffers were used, including 2% skimmed milk and 1% HSA to reduce nonspecific binding.

11.2.5 Determination of the concentration of recombinant HBsAg

Various concentrations of recombinant protein, at concentration 0.5, 1.0, and 1.5 µg/ml in 0.05 M carbonate buffer pH 9.6 (100 µl/well) were used for coating the PolySorp plates, overnight at 4°C. Subsequent steps were performed as 11.2.1.

12. Indirect ELISA for Anti-HBs Antibody Assay

The PolySorp plate was coated with 1.5 µg/ml of recombinant protein in 0.05 M carbonate buffer pH 9.6 (100 µl/well), overnight at 4°C. Between each step, the plates were washed four times in PBS with 0.05% Tween 20 by washing machine. The plates were blocked for 1 hour at 37°C with 200 µl/well of PBS containing 2% skimmed milk (blocking buffer) to reduce nonspecific binding. Serum samples from 41 medical students (pre and post HBV vaccination) and 47 normal person were diluted at 1:100 in blocking buffer, added to the well (100 µl/well), and incubated for 30 minutes at 37°C.

The plates were incubated with peroxidase conjugated anti-human IgG which was diluted 1:10,000 (100 µl/well), for 30 minutes at 37°C. The color was developed by incubation for 30 minutes at room temperature with 10 µg of 3,3´5,5´ tetramethyl benzidine dihydrochloride (TMB) in citrate-phosphate buffer pH 5.0 containing hydrogen peroxide (H_2O_2) (100 µl/well), and the reaction was stopped by addition of 50 µl of 2 N H_2SO_4. The absorbance was read at 492 nm using an automatic microplate reader.

13 Data Analysis

Statistical analysis was performed using the Chi-square test with Yates' collection of Statview 4.5 software (Abacus Concepts, California, USA). Significant difference was considered if the *p* value was less than 0.05.

CHAPTER V

RESULTS

1. The Detection of HBV DNA and Its Nucleotide Sequence in Thai Patients

In this study, a total of 116 HBsAg-positive serum samples were enrolled. The samples were amplified by using sense and anti-sense primers as described in Chapter IV. After the amplification of HBV DNA, PCR products were analyzed by gel electrophoresis on 2% agarose gel stained with ethidium bromide. Visualization of a fragment corresponded to the expected PCR product size (772 bp for *S* gene; 369 bp for the core promoter region and precore region and 257 bp for *C* gene) were considered as positive result, as shown in Figures 6.

In the core promoter region and precore region amplification, 80 out of 116 serum samples could be amplified. Of these, 61 core promoter and precore sequences were further analyzed since 11 samples were repeated samples from the same patients. Sample numbers (1 and 607), (7 and 93), (14 and 652), (18 and 757), (20 and 693), (21 and 745), (22 and 96), (24 and 29), (44 and 92), (50 and 79) and (74 and 99) are from the same patients collected at different time points. Eight samples were unable to be sequenced (No. 70, 72, 85, 100, 105, 107, 759 and c94-60) due to the insufficient amounts or low quality of the sequences obtained. For some samples that could not be amplified using these sets of primers, the presence of HBV DNA genome was confirmed by PCR amplification using another set of primers in the *C* gene.

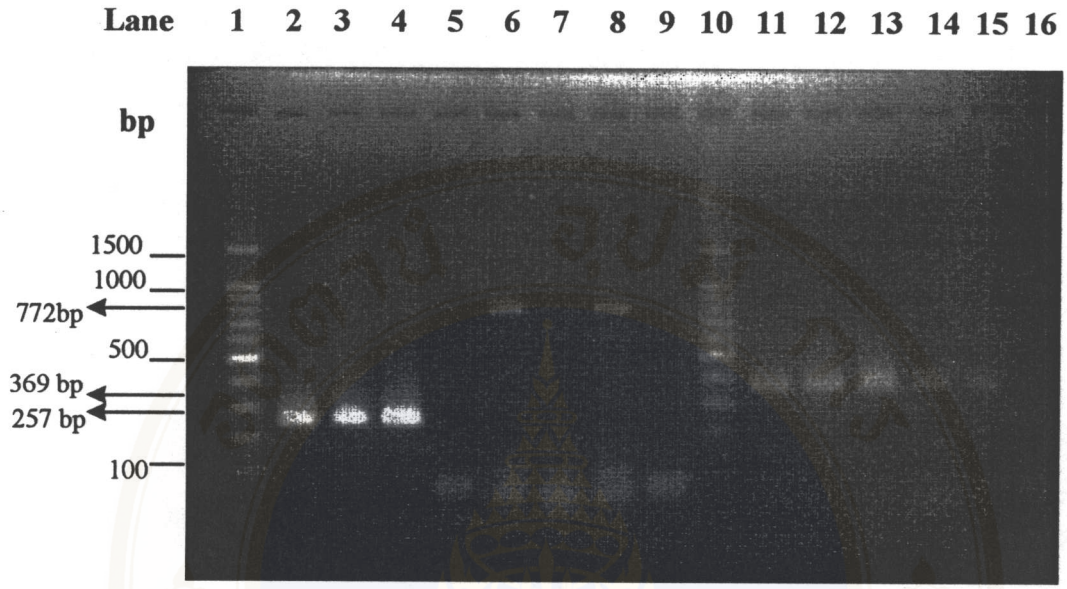


Figure 6. PCR amplification of HBV genes

Lanes 1 and 10: DNA size marker

Lanes 2-5: PCR products of *C* gene from various samples (product size = 257 bp)

Lanes 6-9: PCR products of *S* gene from various samples (product size = 772 bp)

Lanes 11-16: PCR products of the core promoter region and precore region from various samples (product size = 369 bp)

Lanes 5, 7, 9, 16: Negative control

Lanes 4, 8, 15: Positive control

2. DNA Sequence of The Core Promoter Region and Precore Region with Respect to The Viral Genotype

Figure 7 shows phylogenetic tree of 61 the core promoter region and precore region. Genotyping of the obtained HBV core promoter and precore sequences into one of the A-F genotypes was done by the comparison of these sequences with a set of 15 reported isolated with known genotypes; genotype A (Genebank accession number v00866, x02763), genotype B (Genebank accession number d00329, d00330, d23678), genotype C (Genebank accession number m38454, x04615, d23682, d12980, l08805, m12906, d00630), genotype D (Genebank accession number x59795), genotype E (Genebank accession number x75657) and genotype F (Genebank accession number x75663) (Figure 8).

The genotype of each sample was assigned after the phylogenetic analysis.

HBV genotypes A, B and C were found in this study. Of the 61 patients, one was infected with HBV genotype A, 18 with genotype B and 42 with genotype C, with the frequency of 1.6 %, 29.5% and 68.9%, respectively. There were some changes of nucleotides which appeared to be genotype-specific.

The prototype of genotype A has the following nucleotide T1631, C1674, C1703, C1740, C1773, C1802, G1803, A1850, T1915 and A1934, and the sample No. 36 had the same sequence except at position A1727 and T1790.

Prototype genotype B isolate has G1633, A1636, G1652, T1673, C1726, T1727, G1730 and G1799. These positions were found in most of genotype B of Thai isolates, except in No. 51 that had C at position 1673 (C1673) and No. 17 had A at position 1726 (A1726).

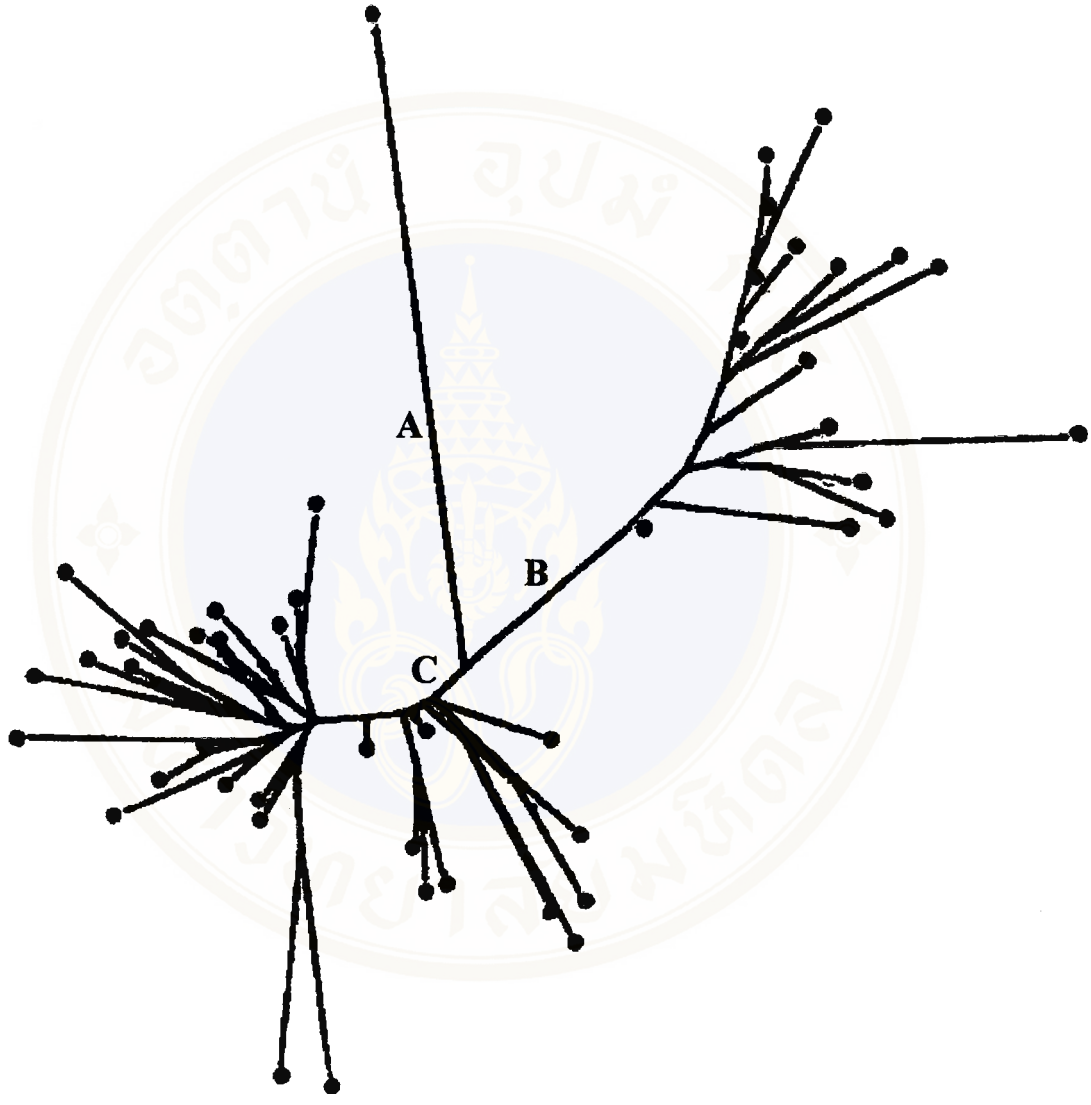


Figure 7. Phylogenetic analysis of Thai isolates based on the nucleotide sequences of the core promoter region and precore/core region. Each circle represents an HBV isolate. The major branches (A, B and C) correspond with the genotypes of HBV.

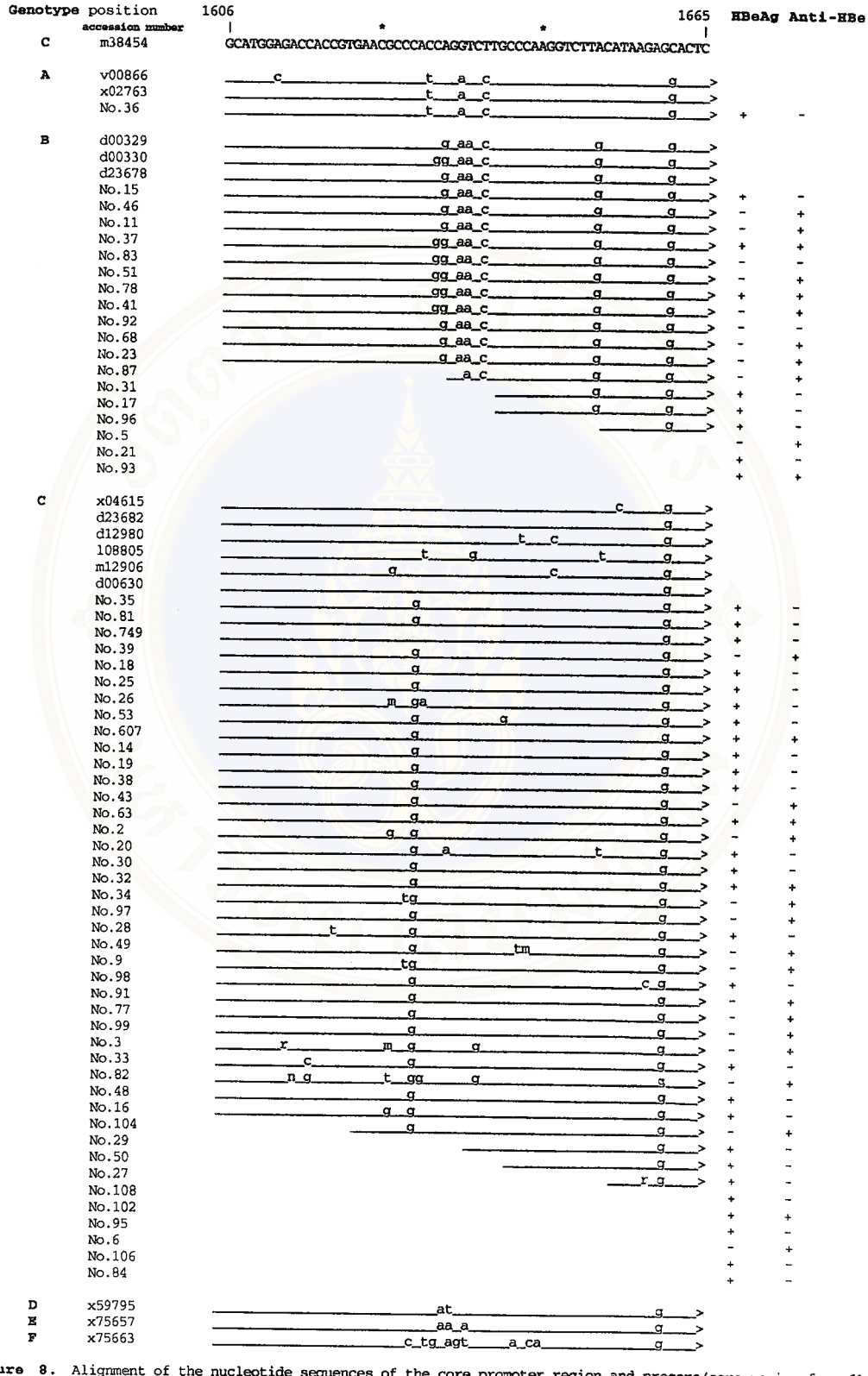


Figure 8. Alignment of the nucleotide sequences of the core promoter region and precore/core region from 61 HBV Thai isolates. Top line is a prototye sequence using nucleotide numbering by Gan *et al* (1987)(58). Line represents nucleotide identical to reference sequence. Deletions are shown as shash (/) line. Insertions, as strainght (|) line. The accession number of the 15 strains represent genotype A to F are shown. The status of HBeAg/anti-HBe of each patient are also shown. Abbreviation: -, negative : +, positive.

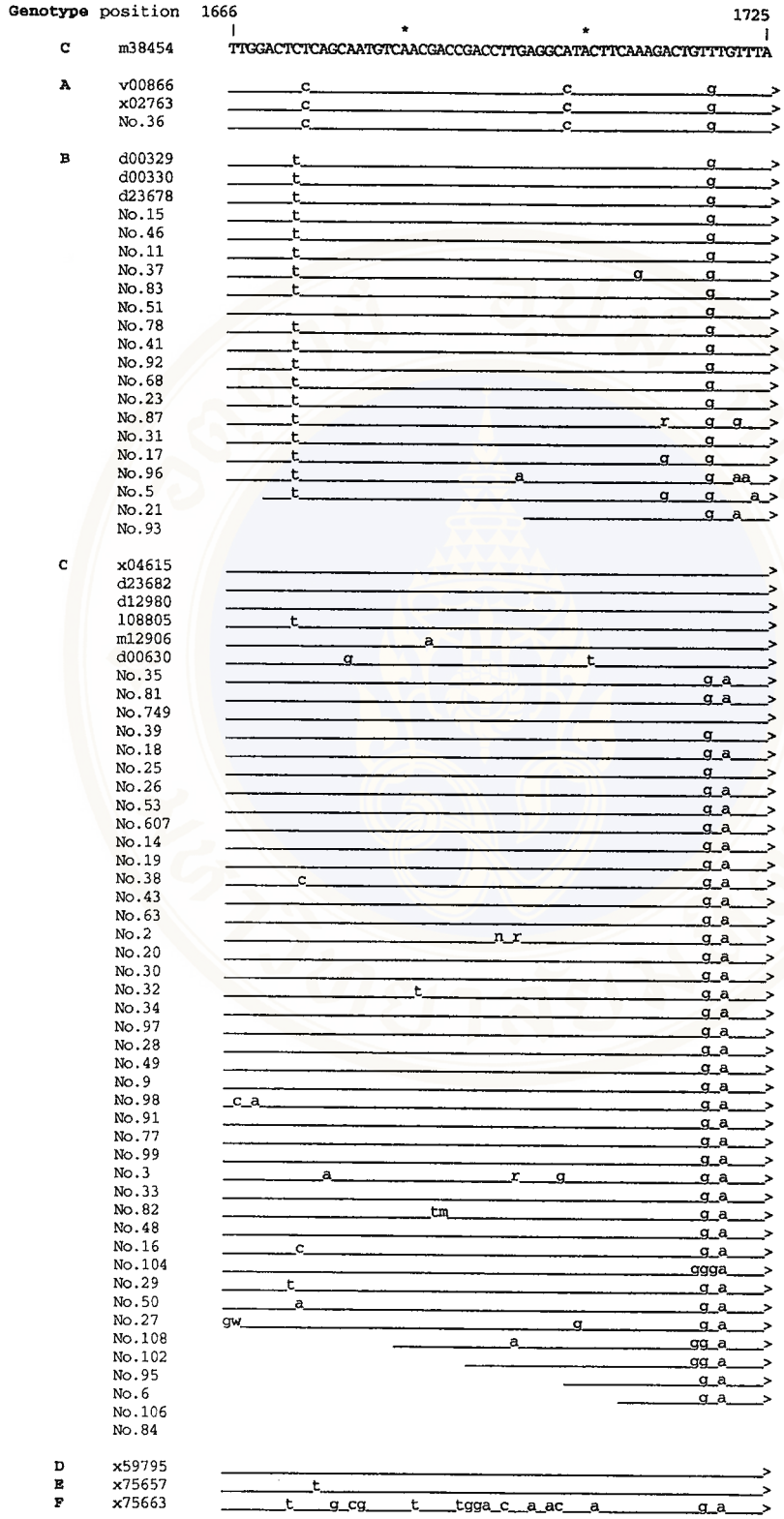
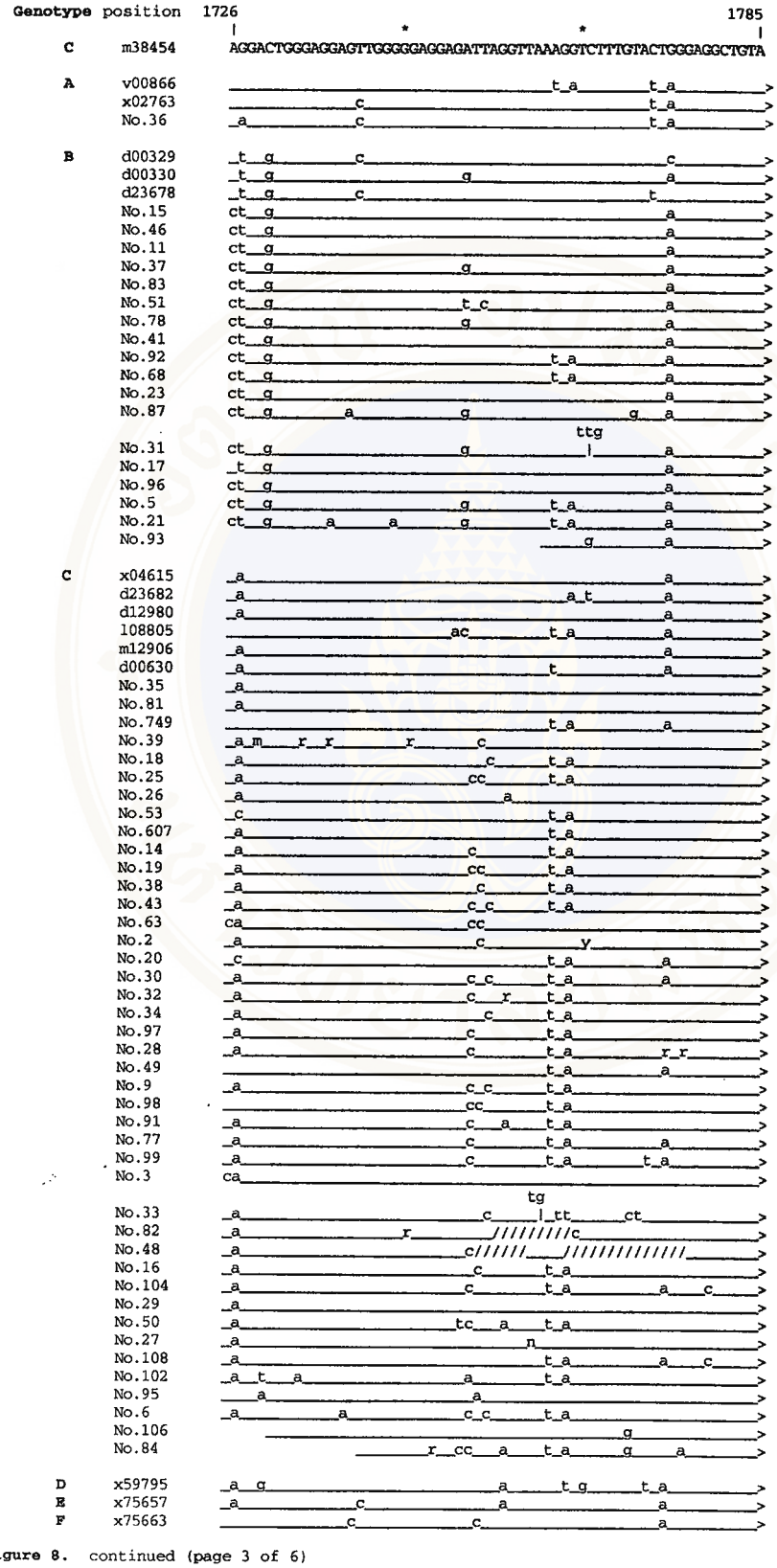


Figure 8. continued (page 2 of 6)



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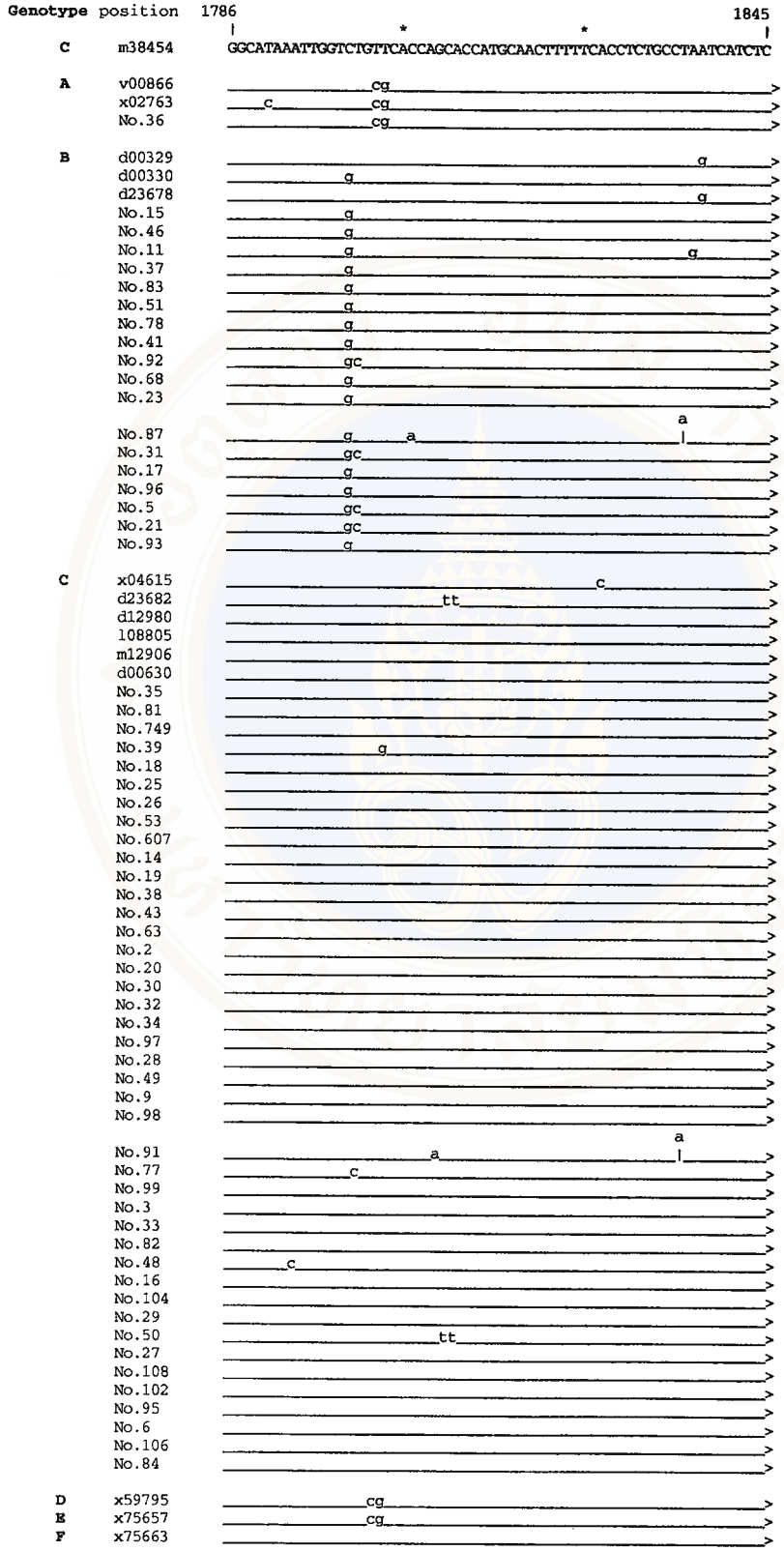


Figure 8. continued (page 4 of 6)

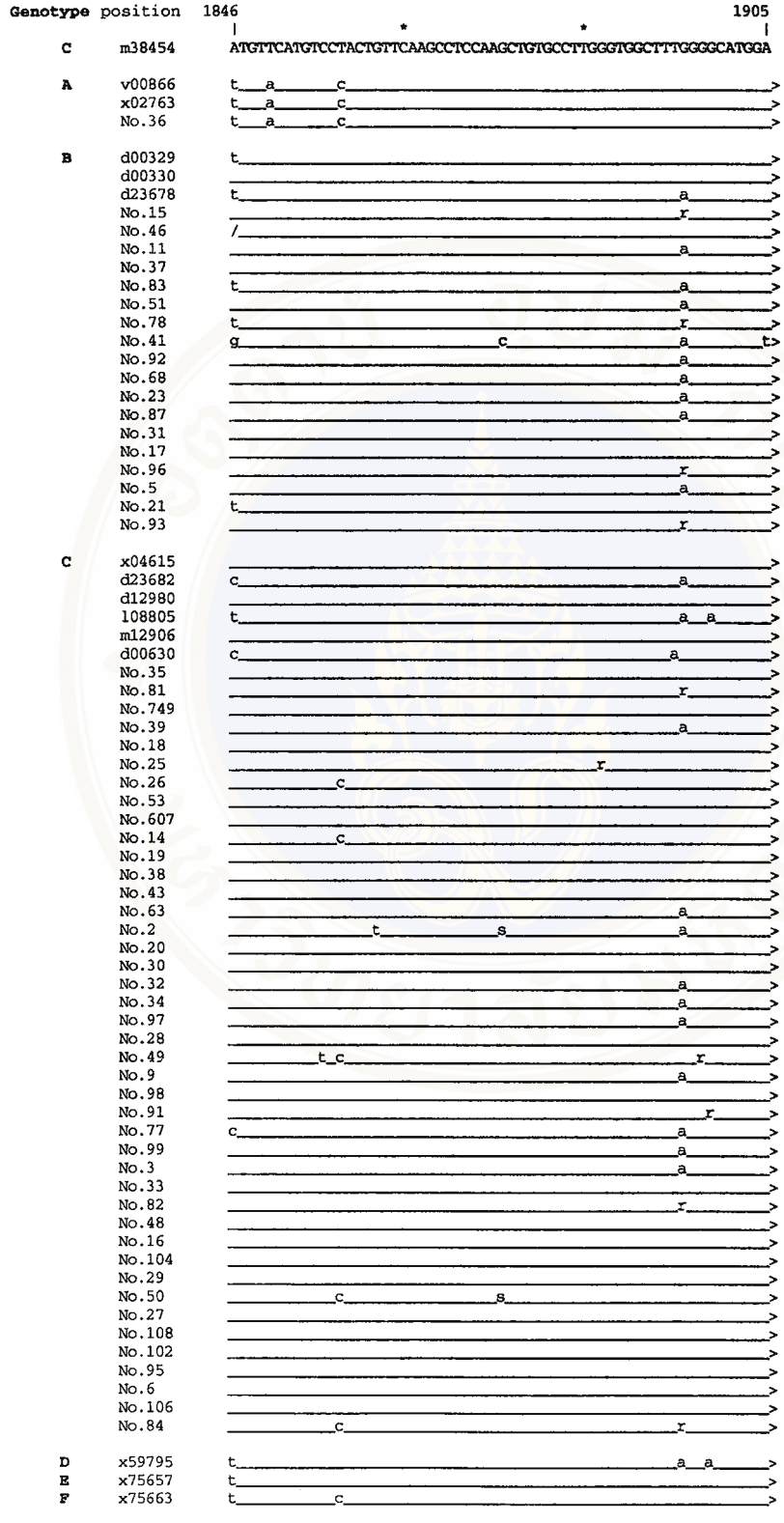


Figure 8. continued (page 5 of 6)

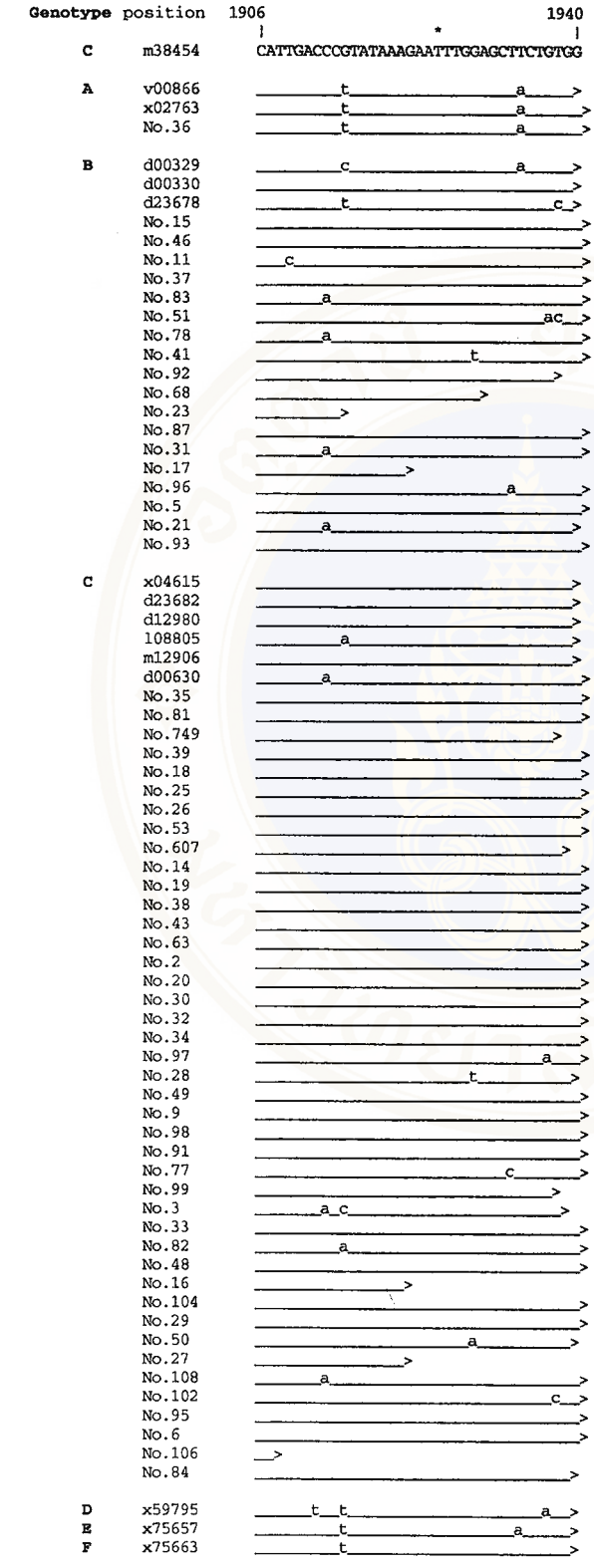


Figure 8. continued (page 6 of 6)

For HBV genotype C of Thai isolates, the nucleotide sequences were similar to that of the prototype isolate (m38454) (58), however, there were four nucleotide positions which were different from other prototype isolates (x04615, d23682, d12980, l08805, m12906, d00630). The nucleotide at position 1630 was G (A1630G) in 32 (97.0%) of 33 Thai isolates (Figure 9). G was present at position 1719 (T1719G) in 39 (97.5%) of 40 Thai isolates (Figure 10), while only one isolate had T at this position. At position 1721 (G1721A), there was A in 37 (92.5%) of 40 Thai isolates and at position 1775 (A1775G) was G in 32 (76.2%) of 42 isolates (Figure 11).

Nucleotide insertion and deletion were found in the core promoter region of HBV Thai isolates (Table 3). These were not found in genotype A. In genotype B, two isolates had nucleotide insertion; No. 87 had one inserted nucleotide (A) at position 1839 (Figure 12) and No. 31 had three inserted nucleotides (TTG) between the position 1767 to 1769 (Figure 13). One isolate (No. 46) had one nucleotide (A) deletion at position 1846 (Figure 14).

For genotype C, there were two isolates with nucleotide insertion. No. 33 had two inserted nucleotides (TT) between at position 1766 to 1767 (Figure 15) and No. 91 had one nucleotide insertion (A) at position 1839 (Figure 12). Two isolates had nucleotide deletion. No. 48 had twenty nucleotide (20 bp) deletion between position 1754 to 1759 and 1764 to 1777 (Figure 16), and the other (No. 82) had nine nucleotides (9 bp) deletetion between position 1756 to 1764 (Figure 17). In summary, 4 isolates had insertion and 3 isolates had deletion in the core promoter region and precore region.

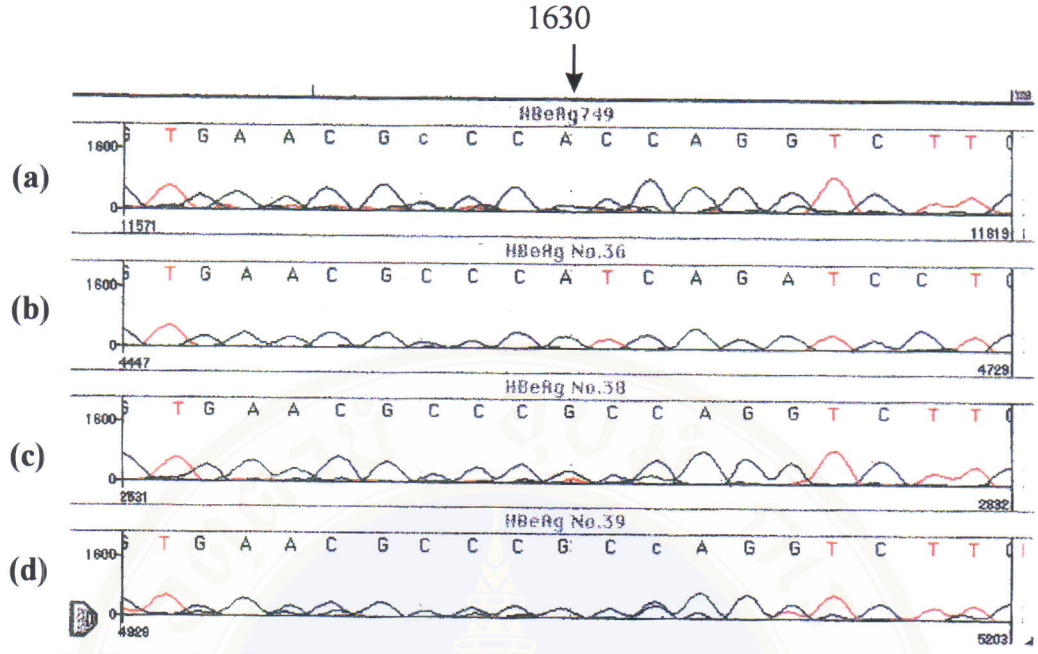


Figure 9. Example of nucleotide sequence variation in the core promoter region. (a), sample No. 749; (b), sample No. 36; (c) sample No. 38; (d), sample No. 39. (a), (c) and (d) are of genotype C whereas (b) is of genotype A. Variation at nt position 1630 is shown. (a) and (b) has A at this position identical to the prototype isolate whereas (c) and (d) has a mutation from A to G (A1630G).

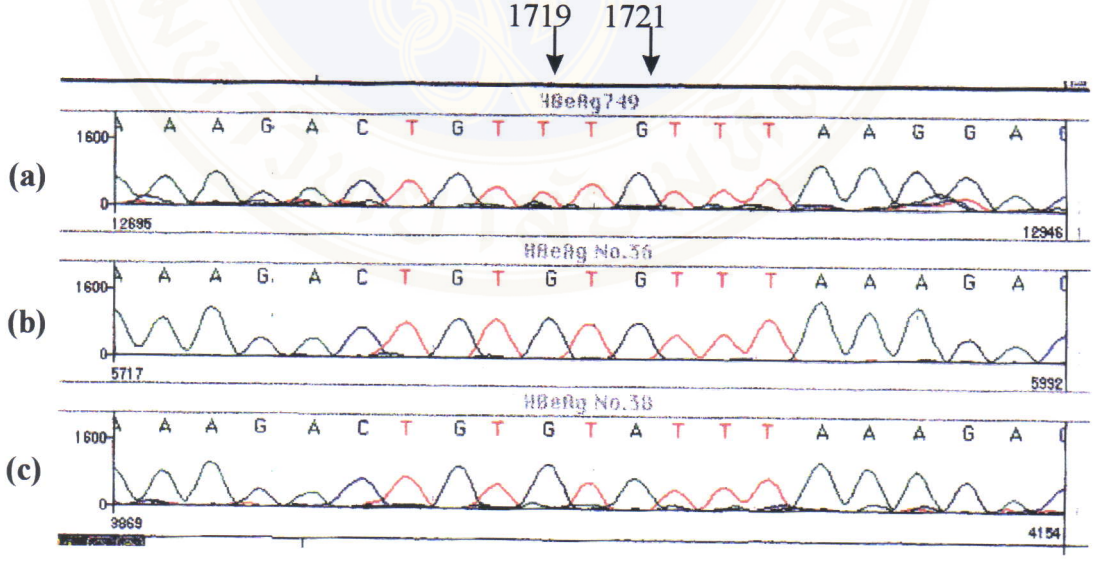


Figure 10. Example of nucleotide sequence variation the core promoter region. (a), sample No. 749; (b), sample No. 36; (c) sample No. 38. (a) and (c) are of genotype C whereas (b) is of genotype A. Variation at nt position 1719 and 1721 are shown. (a) has T at position 1719 (T1719) and G at position 1721 (G1721) identical to the prototype isolate whereas (c) has double mutations from T to G (T1719G) and G to A (G1721A).

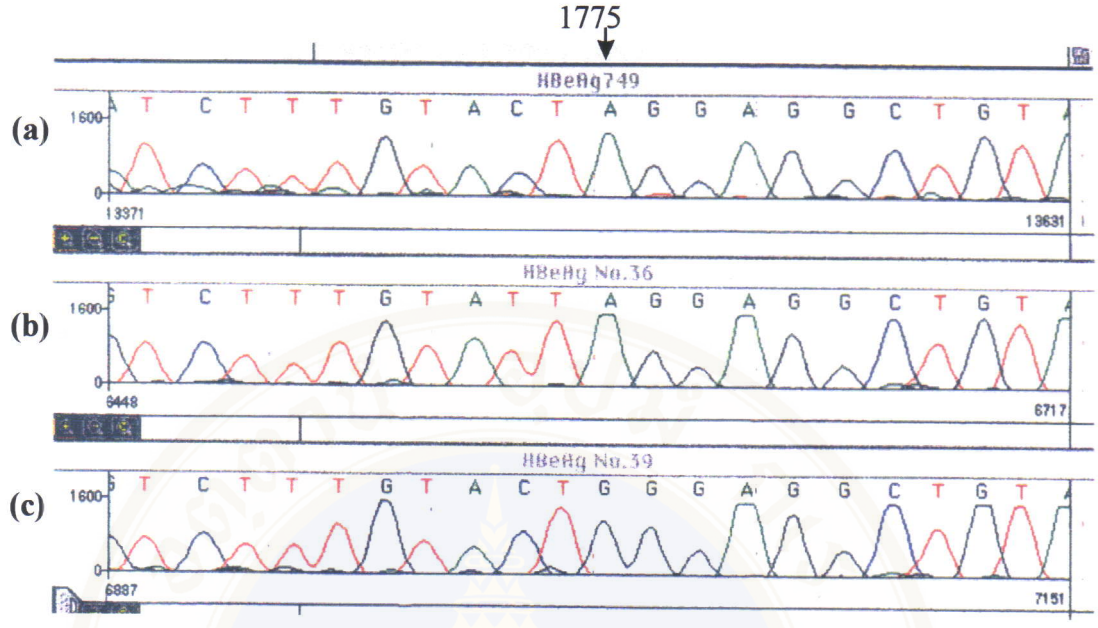


Figure 11. Example of nucleotide sequence variation in the core promoter region. (a), sample No. 749; (b), sample No. 36; (c), sample No. 39. (a) and (c) are of genotype C whereas (b) is of genotype A. Variation at position 1775 is shown. (a) and (b) has A at this position identical to prototype isolate whereas (c) has a mutation from A to G (A1775G).

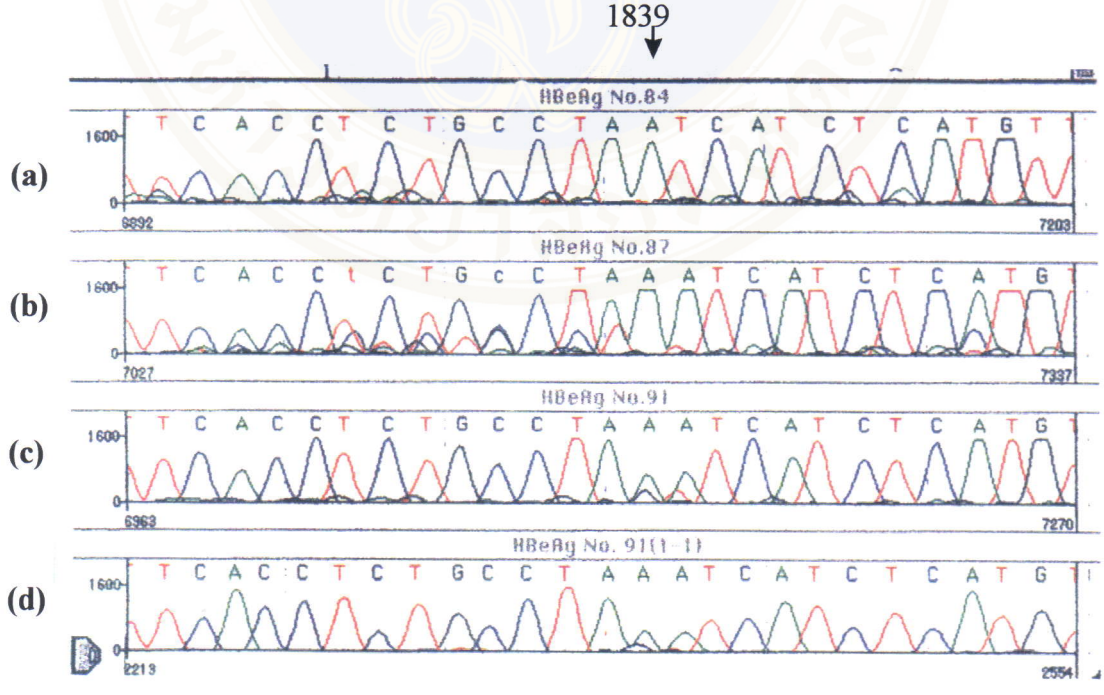


Figure 12. Example of nucleotide sequence variation in the core promoter region, showing a single-base insertion. (a), wild type sequence from sample No. 84; (b), sample No. 87; (c) and (d), sample No. 91, sequenced using sense and antisense primers, respectively. There is a single-base insertion of A at position 1839 in (b), (c) and (d), giving AAAT instead of AAT.

Table 3. Summary of the nucleotide insertion or deletion in the core promoter region found in Thai patients.

Genotype	Sample No	Sequence	Type of mutation	bp	Position
B	87	TAAATCA	insertion	1	1839
	31	GTCTTGTTT	insertion	3	1767-1769
	46	CTCATGT	deletion	1	1846
C	33	GATTTCTT	insertion	2	1766-1767
	91	TAAATCA	insertion	1	1839
	48	GACTAGGTTAAAGATCTTTGTACTGGGAG	deletion	20	1754-1759, 1764-1777
	82	TTAGGTTAAAGGCCT	deletion	9	1756-1764

Underlined sequences represent insertion or deletion

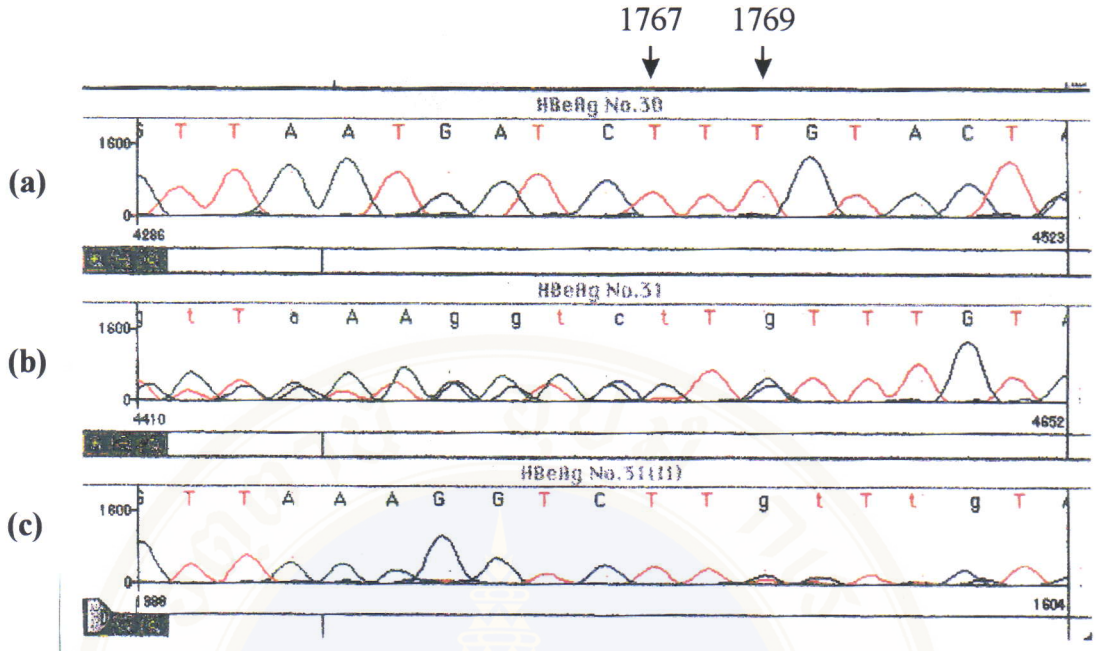


Figure 13. Example of nucleotide sequence variation in the core promoter, showing three-bases (TTG) insertion. (a), wild type sequence from sample No. 30. (b) and (c), sample No. 31, sequenced using sense and antisense primers, respectively. There are three bases (TTG) insertion between position 1767 to 1769 in (b) and (c), giving CTTGTTT instead of CTTT.

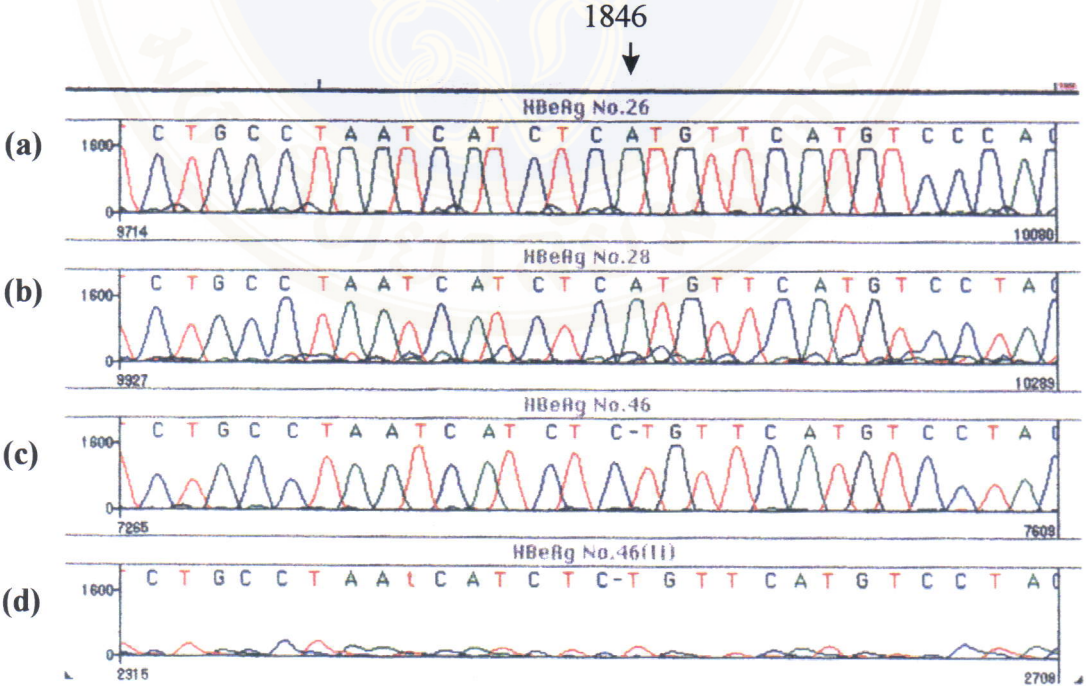


Figure 14. Example of nucleotide sequence variation in the core promoter region, showing a single-base deletion. (a) and (b), wild type sequences from sample No. 26 and No. 28. (c) and (d), sample No. 46, sequenced using sense and antisense primers, respectively. There is a deletion of A at position 1846 in sample No. 46, resulting in CT instead of CAT.

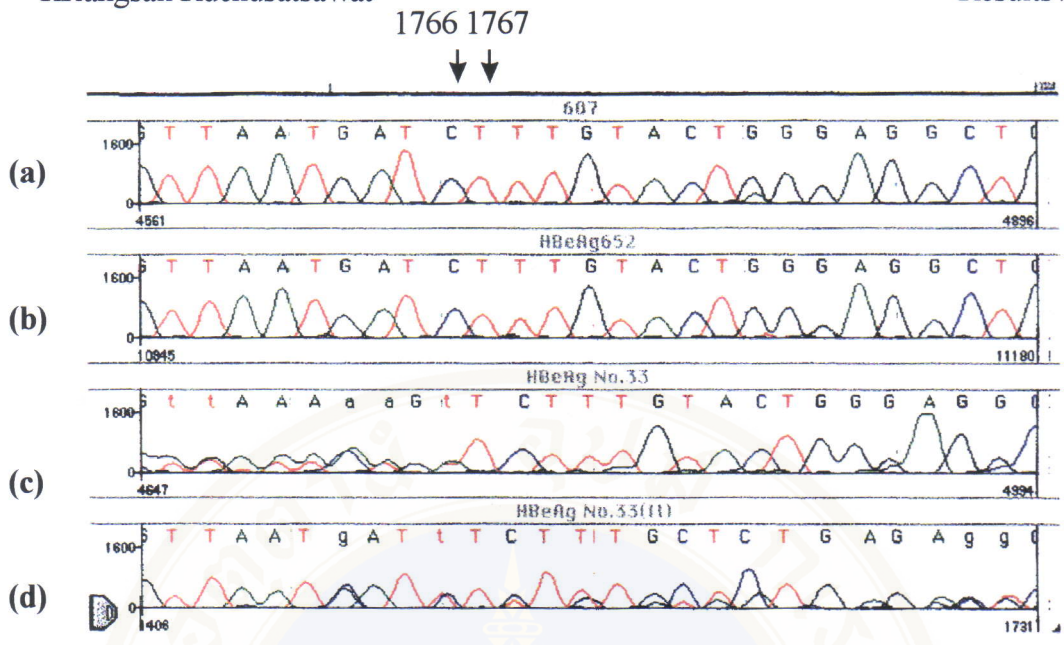


Figure 15. Example of nucleotide sequence variation in the core promoter region, showing two-bases (TT) insertion. (a) and (b), wild type sequences from sample No. 607 and No. 652. (c) and (d), sample No. 33, sequenced using sense and antisense primers, respectively. There are two bases (TT) insertion between position 1766 to 1767 in (c) and (d), giving GATTTCT instead of GATCT.

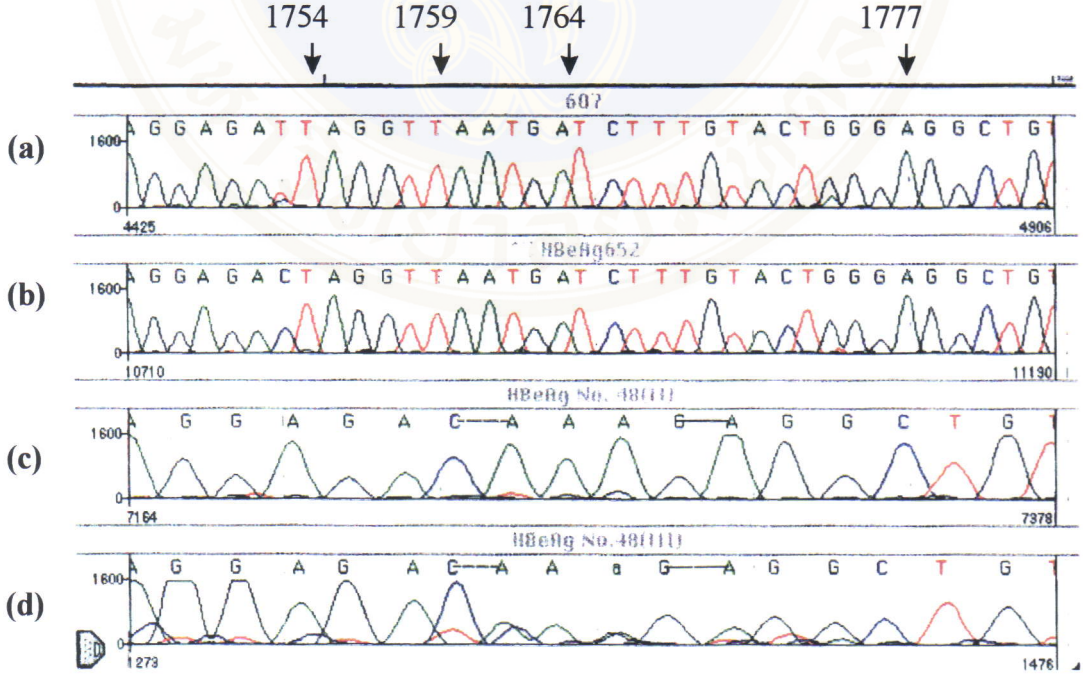


Figure 16. Example of nucleotide sequence variation in the core promoter region, showing twenty-bases deletion. (a) and (b), wild type sequence from sample No. 607 and No. 652. (c) and (d), sample No. 48, sequenced using sense and antisense primers, respectively. There are twenty bases deletion between position 1754 to 1759 and 1764 to 1777 in sample No. 48, resulting in GACAAAGAGG instead of GACTAGGTTAAAGATCTTTGTACTGGG AGG.

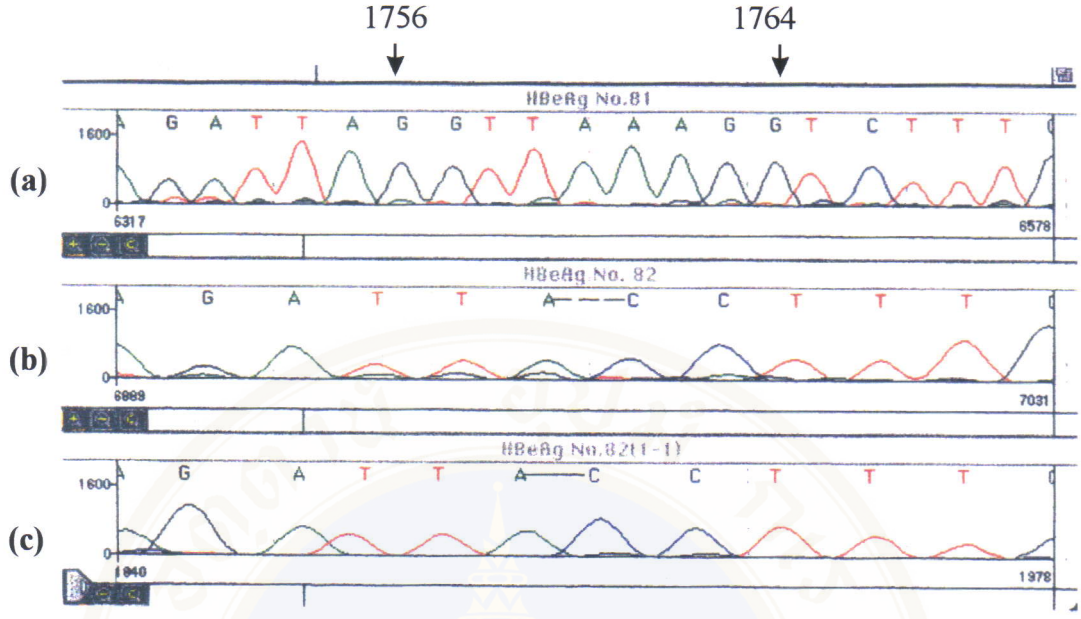


Figure 17. Example of nucleotide sequence variation in the core promoter region, showing nine-bases deletion. (a), wild type sequence from sample No. 81 (b) and (c), sample No. 82, sequenced using sense and antisense primers, respectively. There are nine bases deletion between position 1756 to 1764 in sample No. 82, resulting in TTACCT instead of TTAGGTTAAAGGCCT

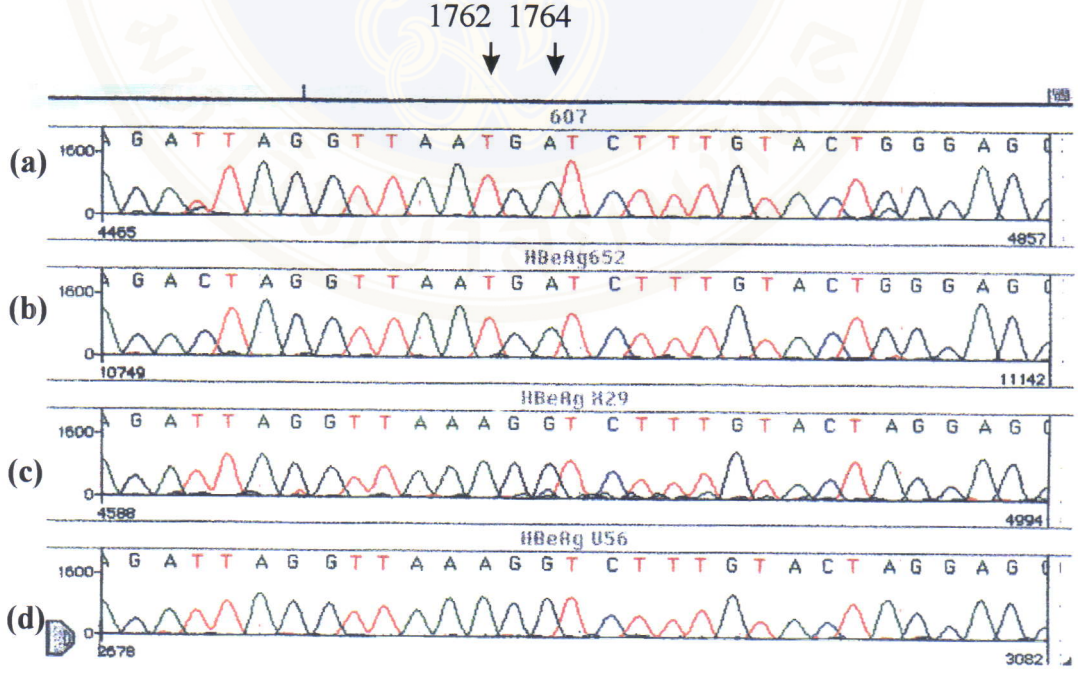


Figure 18. Example of nucleotide sequence variation, showing double mutation at position 1762 and 1764 in the core promoter region. There is a double mutation at position 1762 (A1762T) and 1764 (G1764A) in (a), sample No. 607 and (b), sample No. 652 compared with wild type sequences in (c), sample U56 and (d), sample X29.

There is also a variable region between nucleotide 1751 to 1757 (1751GATTAGG1757) in the core promoter sequence. In genotype B, A1752G was found in 6 of 17 isolates and T1754C was found in one isolate (No. 51). For genotype C, A1752G was found in one isolate and A1752T was found in one isolate. T1753C was found in 19 isolates and T1753A was found in one isolate. At position 1754, T1754C was found in 8 isolates and T1754A was found in one isolate. At position 1755, A1755C was found in 7 isolates and G1757A was obtained from 4 isolates, one had G1757r. In additional, C1653T was found in one isolate (No. 20).

3. Correlation of Nucleotide at Position 1762 (A1762T), 1764 (G1764A) and Precore Region with HBeAg Status

An A to T substitution at nucleotide 1762 (A1762T) and G to A at nucleotide 1764 (G1764A) was found in 4 (22.2%) of 18 isolates of genotype B, 29 (66.7%) of 42 isolates of genotype C and none of genotype A (Figure 18). In all, this double mutation was found in 33 (54.1%) of 61 samples and is the most common type of mutation.

Five mutations in the precore region were found; a nucleotide substitution C to T at position 1856 (C1856T), T1858C (Figure 19), G1896A (Figure 20), G1898r (Figure 21) and G1899r (Figure 22). Double C1856T, G1898r mutations were found in one isolate (No. 49), and G1988r was found in No. 91. At position 1858, T1858C was found in one isolate of genotype A (No. 36), 5 isolates of genotype C (No. 14, 26, 49, 50, 48) and none of genotype B.

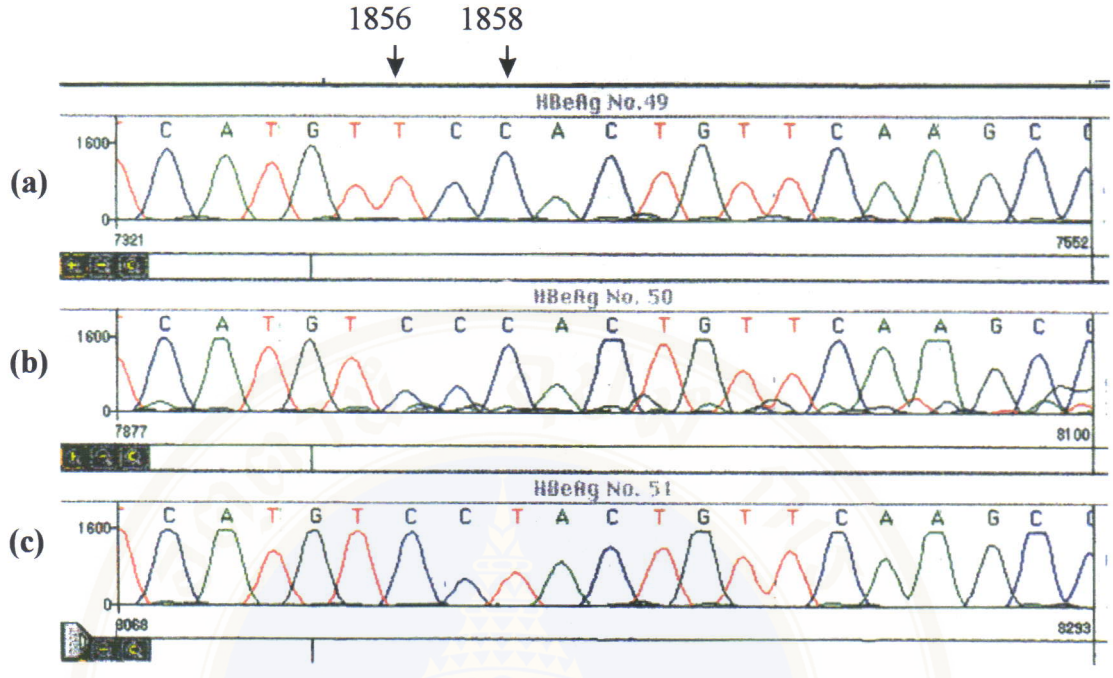


Figure 19. Example of nucleotide sequence variation in the precore region. There is a double mutation at position 1856 (C1856T) and 1858 (T1858C) in (a), sample No. 49, and only a single mutation at position 1858 (T1858C) in (b), sample No. 50 compared with wild type in (c), sample No. 51.

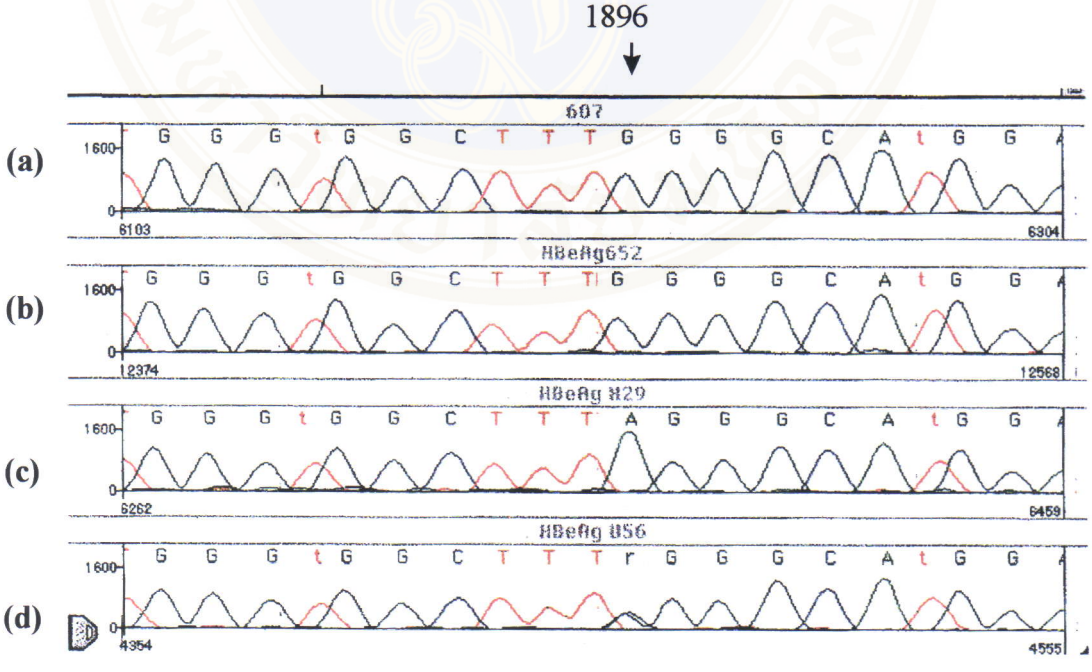


Figure 20. Example of nucleotide sequence variation in the precore region. There is a single mutation at position 1896 (G1896A) in (c), sample X29 compared with wild type in (a), sample No. 607 and (b), sample No. 652. (d), sample U56 showed mixed infection (G1896r) with both the mutant and wild type viruses.
Note: r = A or G

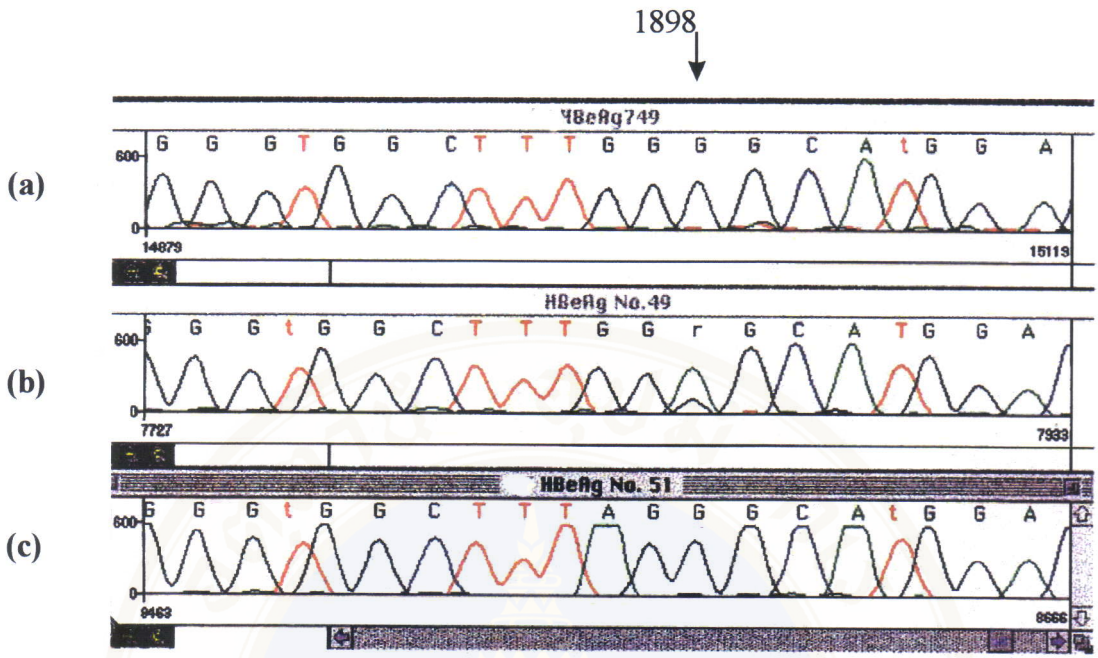


Figure 21. Example of nucleotide sequence variation in the precore region. There is a single mutation at position 1898 (G1898A). (b), sample No. 49 shown mixed infection (G1898r) with both wild type and mutant viruses, compared with wild type in (a), sample No. 749 and (c), sample No. 51.
Note: r = A or G

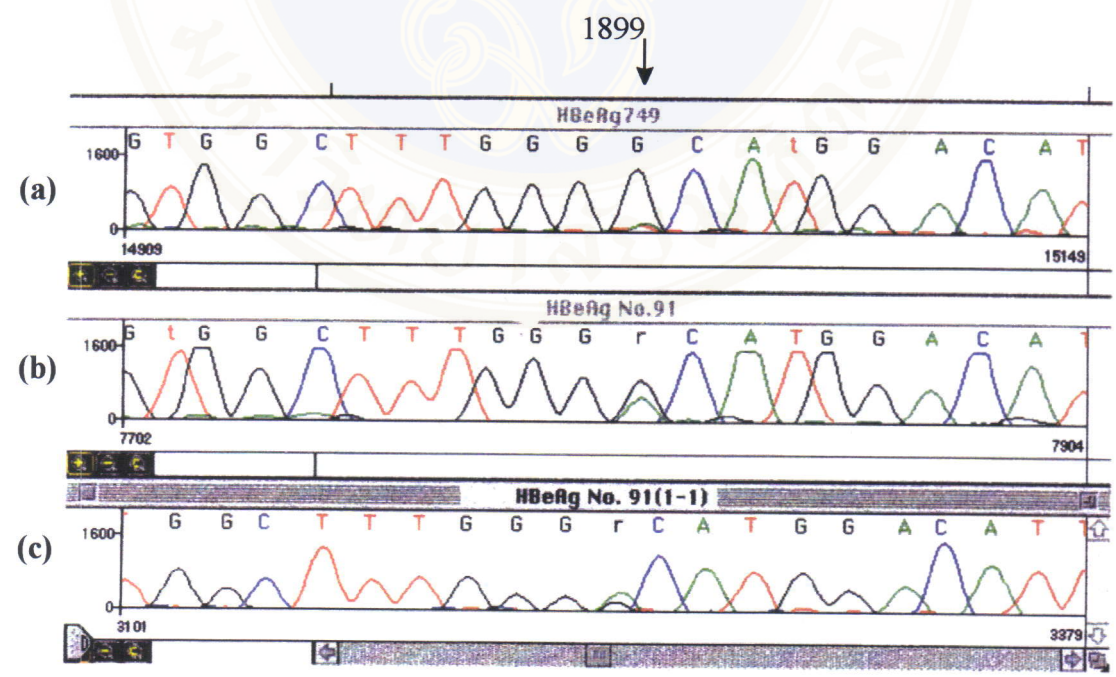


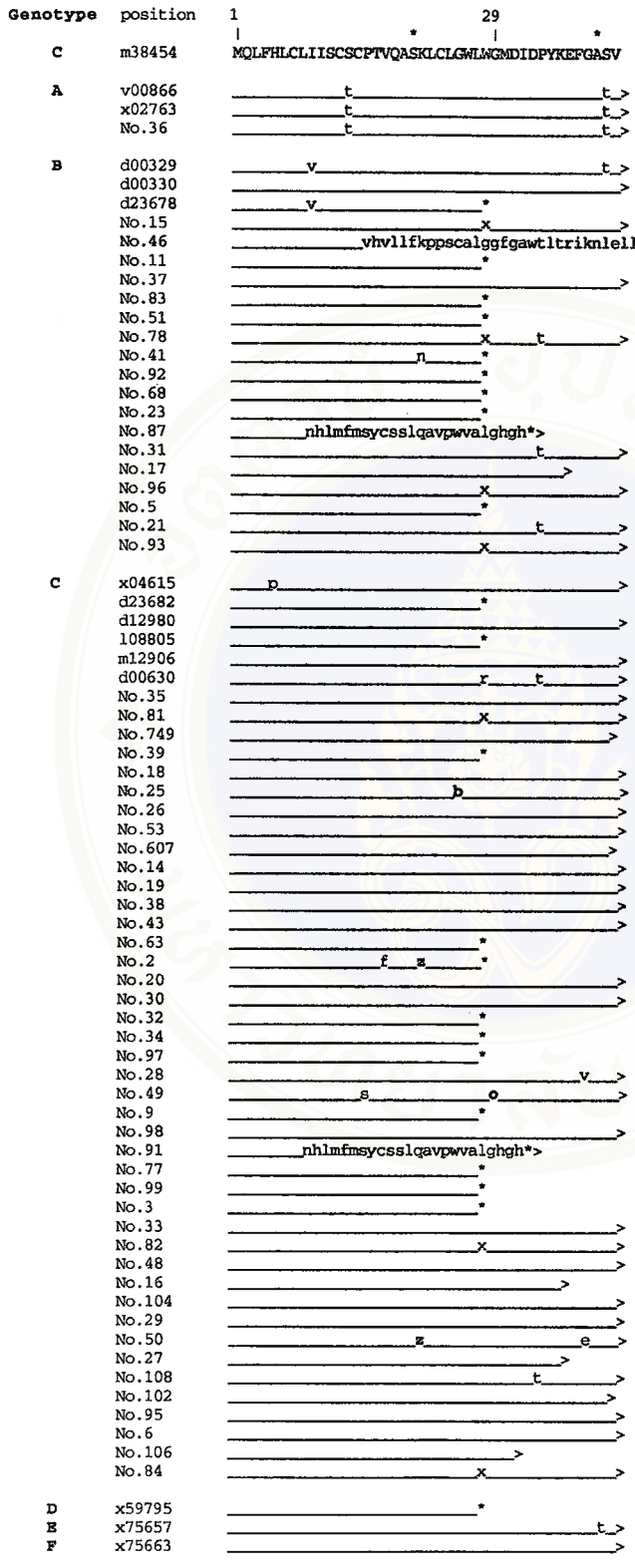
Figure 22. Example of nucleotide sequence variation in the precore region. There is a single mutation at position 1899 (G1899A). (b) and (c), sample No. 91 sequenced using sense and anti-sense primers, respectively. They shown mixed infection (G1899r) with both wild type and mutant viruses, compared with wild type in (a), sample No. 749.
Note: r = A or G

The mutation at position 1896 (TAG) creating a stop codon at codon 28 was detected in 9 (50%) of 18 isolates of genotype B, and 10 (23.8%) of 42 isolates of genotype C. At the same position, mixed infection (TrG) between the wild type (TGG) and the mutant (TAG), was found in 4 (22.2%) of 18 isolates of genotype B, and 3 (7.1%) of 42 isolates of genotype C, while a single isolate of genotype A had no mutation.

Table 4 showed the correlation of nucleotide at position A1762T, G1764A and G1986A with HBeAg status. The presence of HBeAg-negative phenotype of genotype B were found in 3 (75%) of 4 isolates at position 1762 and 1764, 9 (100%) of 9 isolates at position G1896A, but none (0%) of 4 isolates at position G1896r. For genotype C, this HBeAg-negative phenotype were found in 10 (34.5%) of 29 isolates at position 1762 and 1764, 8 (80.0%) of 10 isolates at position G1896A, but one (33.3%) of 3 isolates at position G1896r.

The sequences of precore region and the amino acid sequences which were compared with each others are showed in Figure 23.

Most of patients in this set of samples were chronic hepatitis. At position 1762 and 1764, wild type (A1762, G1764) and mutant (T1762, A1764), were analyzed. This mutation was found in 33 (54.1%) of 61 patients. Twenty patients were HBeAg positive, 13 patients were HBeAg-negative. At position 1896, G1896A was found in 19 (31.1%) of 61 patients. Two of 19 patients with mutant virus were HBeAg-positive, and 17 of 19 were HBeAg-negative. G1896r which was a mixed infected with wild type and mutant , was found in 7 patients (11.5%) and all but one (No. 82 was HBeAg-negative) were HBeAg-positive.



* = stop codon
x = nucleotide mixed between A or G, at codon 28 resulting in stop codon or W
b = nucleotide mixed between A or G, at codon 25 resulting in E or G
o = nucleotide mixed between A or G, at codon 29 resulting in S or G
z = nucleotide mixed between A or G, at codon 21 resulting in K

Figure 23. Alignment of the deduced amino acid sequences in the precore region of 61 HBV Thai isolates.

Table 4. The correlation between the mutations at positions 1762, 1764, 1896 and the HBeAg status.

	1762/1764 double mutation		G1896A mutation		G1896r mutation (mixed infection)	
	HBeAg+	HBeAg-	HBeAg+	HBeAg-	HBeAg+	HBeAg-
Genotype A	0/0	0/0	0/0	0/0	0/0	0/0
Genotype B	1/4	3/4	0/9	9/9	4/4	0/4
Genotype C	19/29	10/29	2/10	8/10	2/3	1/3
Total	20/33	13/33	2/19	17/19	6/7	1/7

(+) = Positive, (-) = Negative

r = A or G

4. Nucleotide Sequencing of *S* Gene

10 PCR products from *S* gene were purified from agarose gel and used as templates for sequencing as described in Chapter IV. The *S* genes were compared with the published isolates of known genotypes; genotype A (Genebank accession number v00866, x02763), genotype B (Genebank accession number d00329, d00330, d23678), genotype C (Genebank accession number m38454, x04615, d23682, d12980, l08805, m12906, d00630), genotype D (Genebank accession number x59795), genotype E (Genebank accession number x75657) and genotype F (Genebank accession number x75663) and also compared with each other (Figure 24). The putative amino acid sequences were deduced from the *S* genes and are aligned as shown in Figure 25.

The overall similarly of nucleotides sequence (93.0-99.0%) and amino acid sequence (88.9-99.6%) were found when compared with each other. Of those 10 samples, 8 HBV DNA were identified as subtype *adr* (80%) in genotype C (No. 749, 757, 694, 693, 607, 675, 759 and 652) and 2 HBV DNA were subtype *adw* (20%) in genotype B (No. c94-60 and 745). The sequence at positions 121 to 147 (27 aa) represent the 'a' determinant. There were only 4 positions (amino acid residues 126, 131, 134 and 143) that were variable in these 10 Thai HBV samples.

The residues at position 126 were threonine in 7 isolates (No. 694, 693, 607, 675, c94-60, 745 and 652), and isoleucine in 3 isolates (749, 757 and 759). At position 131, all but one (No. 607 which was asparagine) were threonine. Phenylalanine was present at position 134 in 8 isolates, while the other two isolates had tyrosine at this position. At position 143, there was serine in 9 isolates (No. 749, 757, 694, 693, 607, 675, 759, c94-60 and 652) and threonine in one isolate (No. 745).

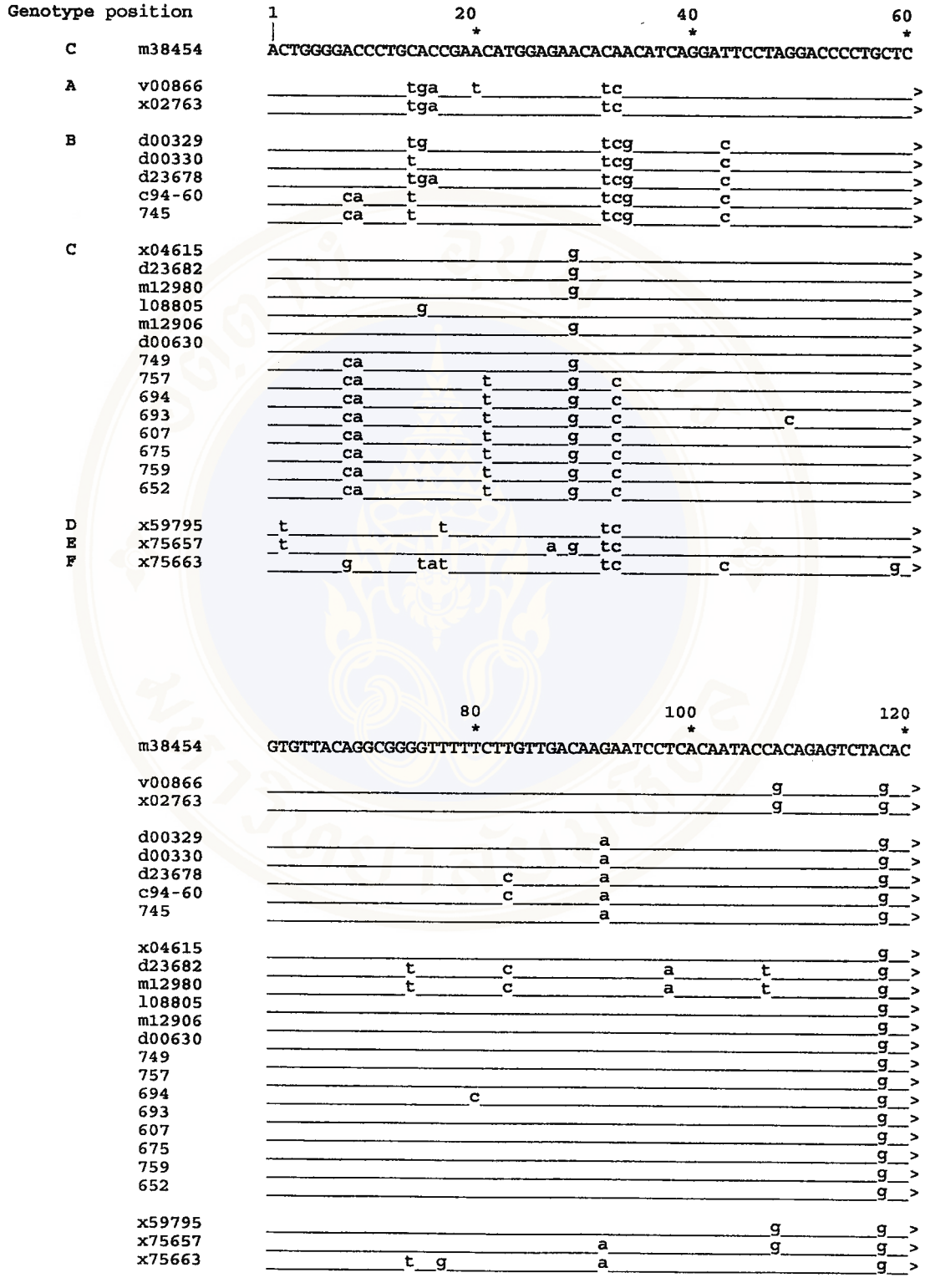


Figure 24. Alignment of the nucleotide sequences of S gene from 10 HBV Thai isolates.

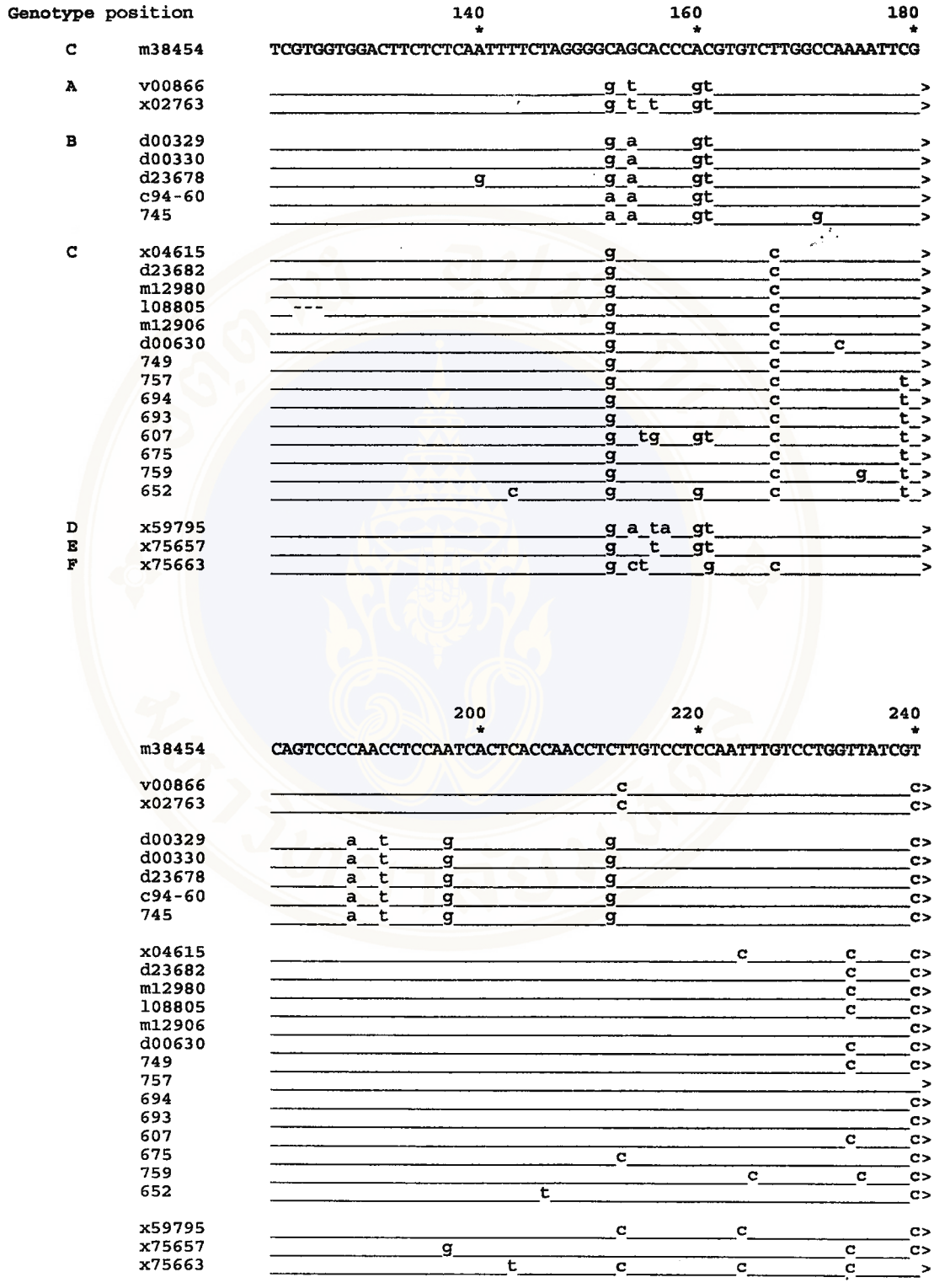


Figure 24. continued (2 of 6)

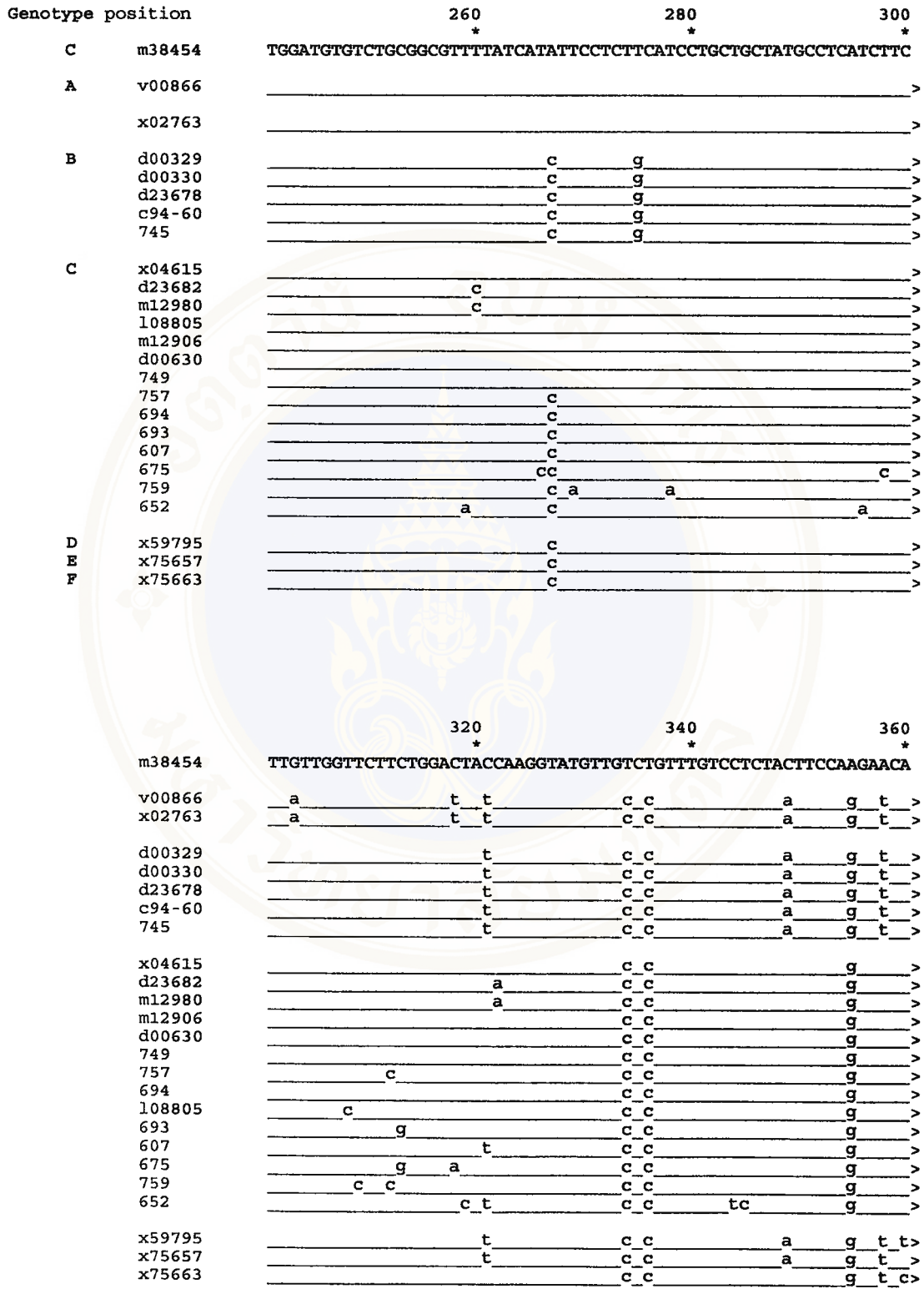


Figure 24. continued (3 of 6)

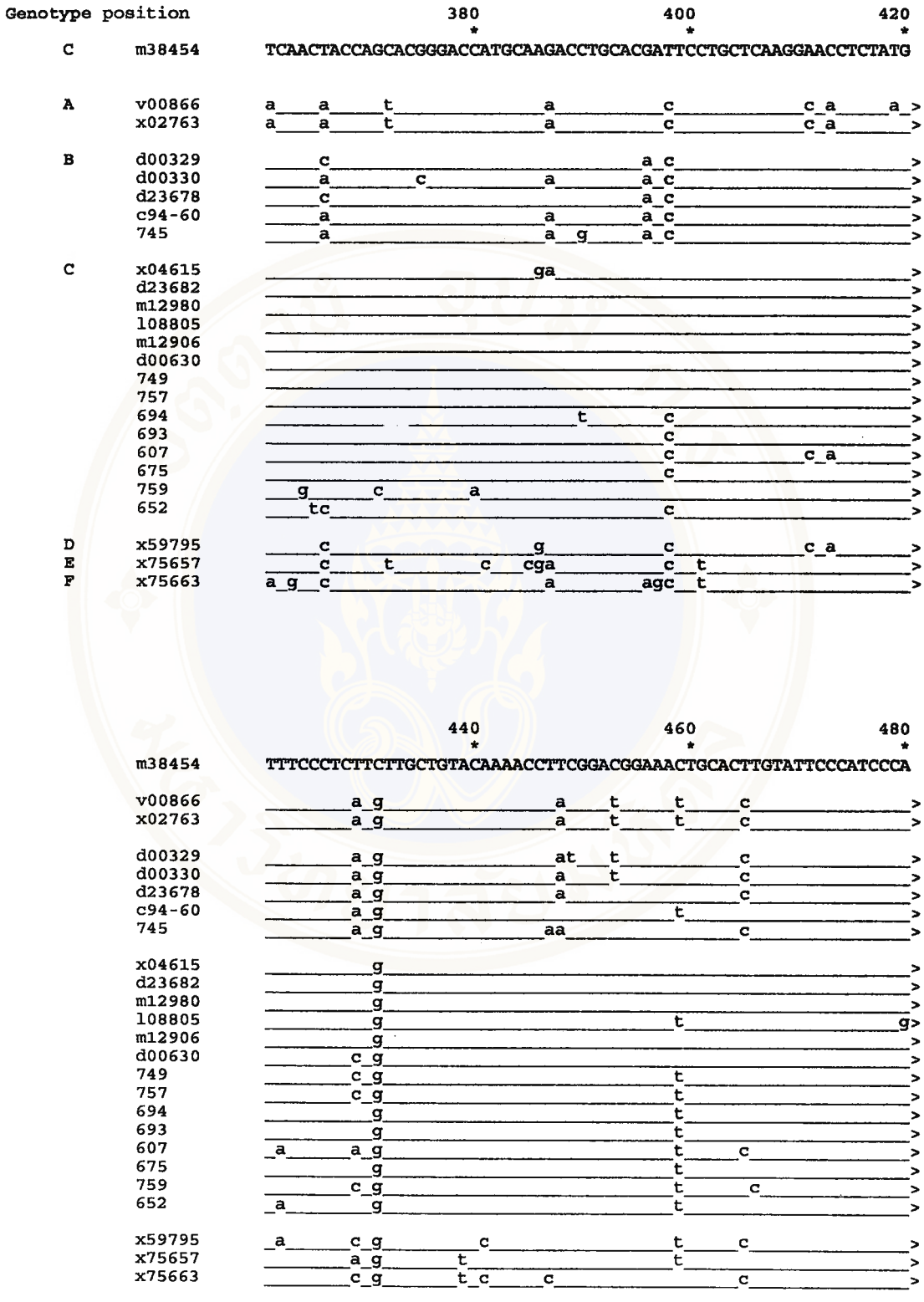


Figure 24. continued (4 of 6)

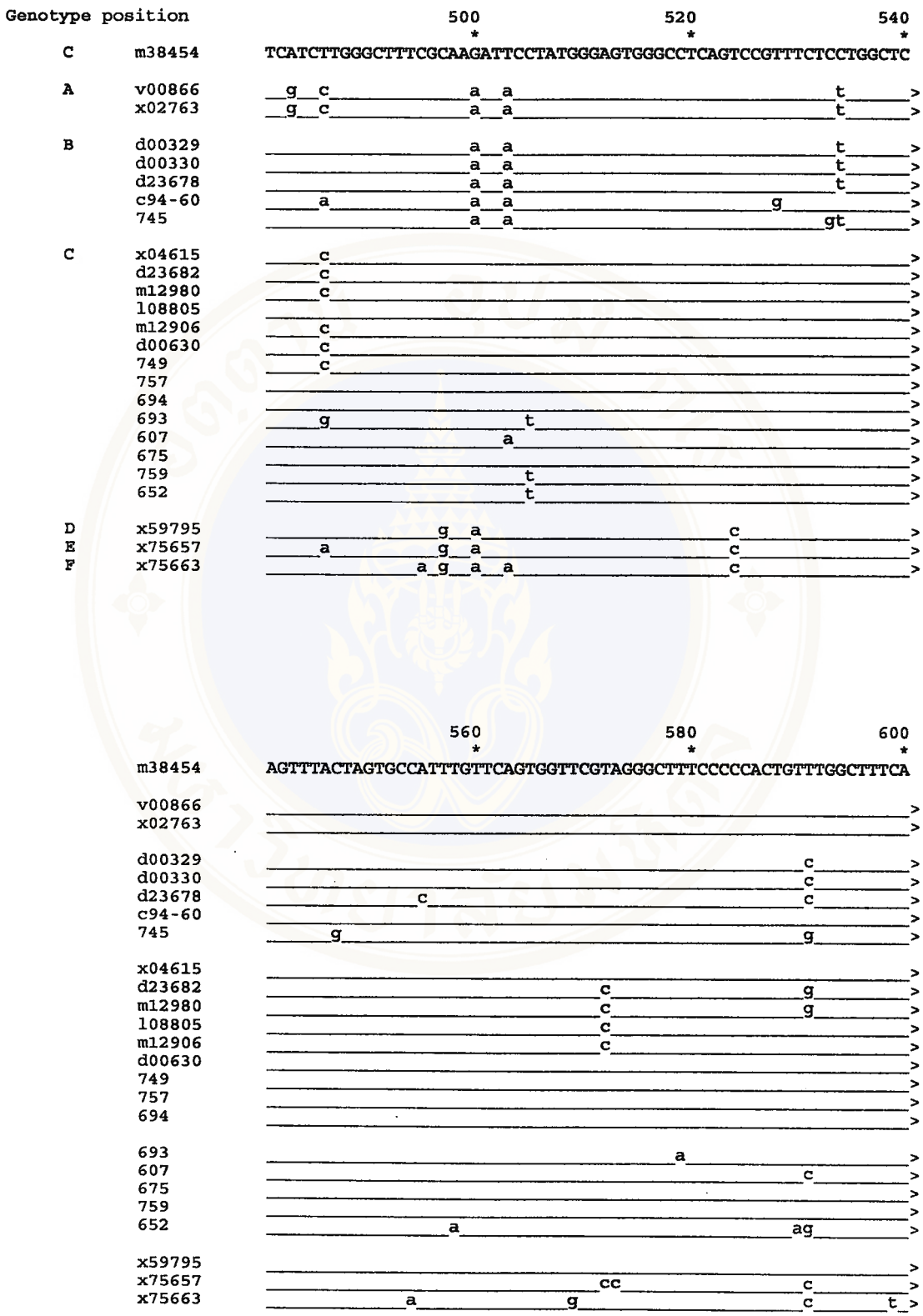


Figure 24. continued (5 of 6)

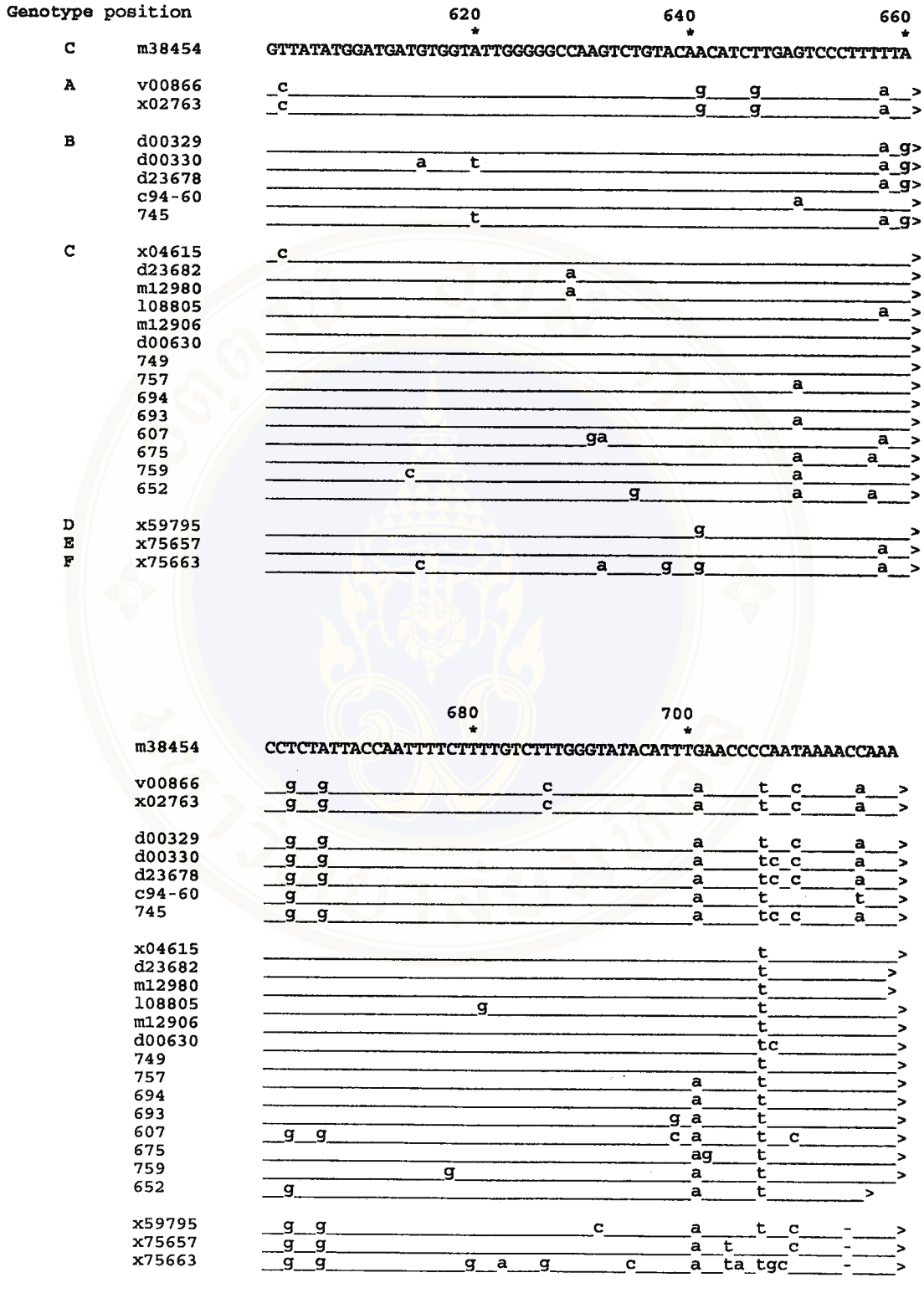


Figure 24. continued (6 of 6)

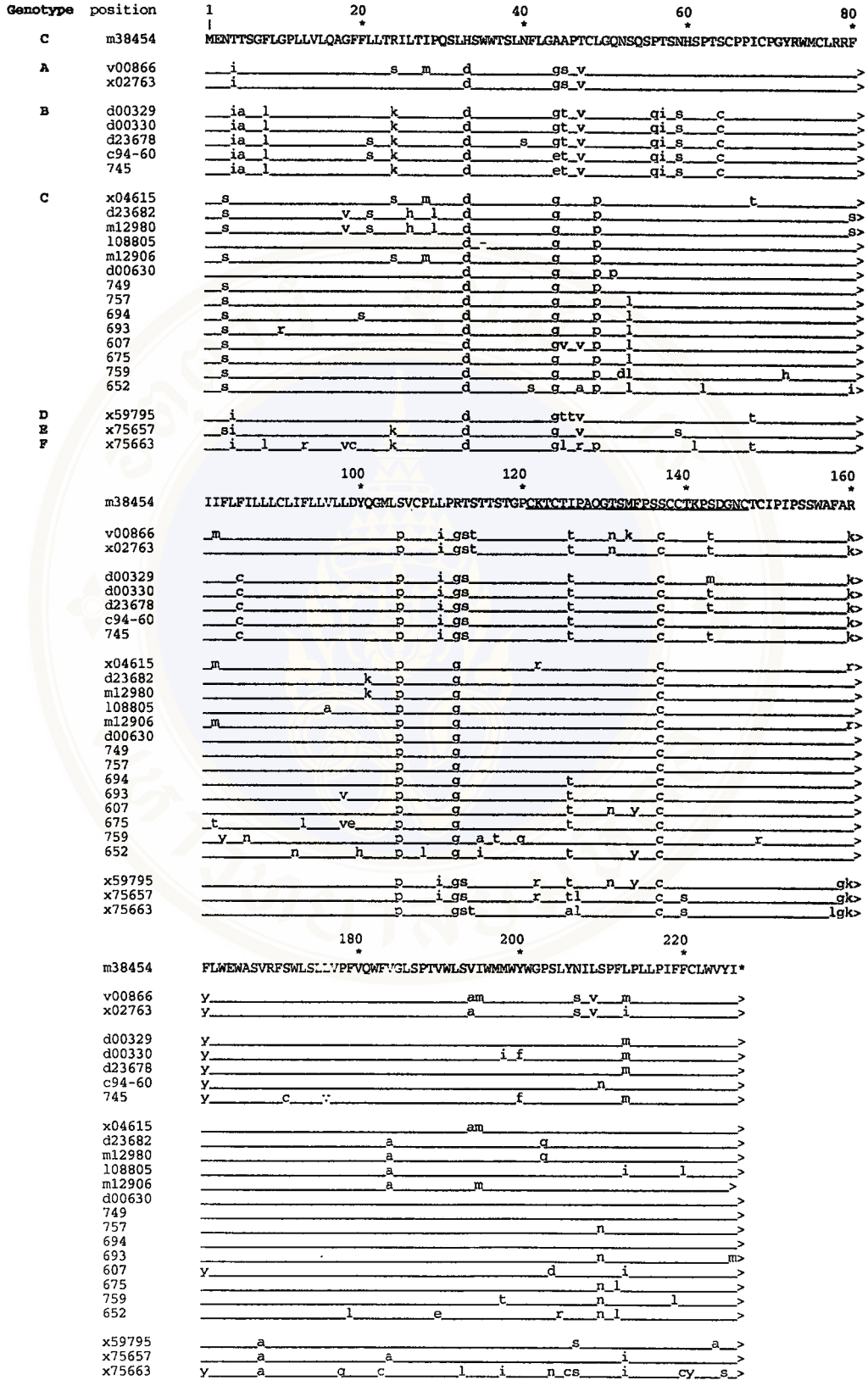


Figure 25. Alignment of the deduced amino acid sequences of the *S* genes from 10 HBV Thai isolates. The 'a' determinant is underlined.

5. Enzyme Immunoassay for Detecting Anti-HBs antibody

Synthetic peptide was designed from the sequences of 10 Thai isolates in the region corresponding to 'a' determinant. The sequence is CKTCTTPAQGTSMFPS CCCTKPSDGNC containing 27 aa in length. This peptide was analyzed as a potential antigen for ELISA using recombinant yeast-derived HBsAg as a control.

5.1 Types of microtiter plate

The effect of microtiter plate surface for binding in ELISA procedure was investigated. For the synthetic peptide, MaxiSorp and Immunlon II showed absorbance of negative control serum (NC) higher than positive control serum (PC), whereas only PolySorp showed absorbance of PC higher than NC. The result for recombinant HBsAg was similar (Table 5). The PolySorp microtiter plate was used in further experiments.

Table 5. Optimization of microtiter plate.

Antigen	MaxiSorp	PolySorp	Immulon II
Synthetic peptide			
PC	0.557	0.124	0.642
NC	0.880	0.070	0.826
Recombinant HBsAg			
PC	0.319	0.806	0.301
NC	1.353	0.499	0.430

5.2 Dilution of serum

In order to determine the optimal dilution of serum, for synthetic peptide, positive and negative control sera were diluted with 2% skimmed milk. Dilution of both sera at 1:10 give higher absorbance than 1:100 and 1:1000. For recombinant HBsAg, positive and negative control sera were diluted with 2% skimmed milk. The dilution at 1:10 give higher absorbance than 1:100 but dilution of 1:1000 was not done (Table 6). The 1:100 dilution gave good discrimination between the positive and negative controls.

Table 6. Dilution of serum.

Dilution of serum	1:10	1:100	1:1000
Synthetic peptide			
PC	0.262	0.194	0.132
NC	0.153	0.052	0.015
Recombinant HBsAg			
PC	0.870	0.510	nd
NC	0.453	0.198	nd

5.3 Various concentrations of conjugate

For peptide, dilution of enzyme conjugated anti-human IgG at 1:20,000 gave lower absorbance than at 1:10,000 when tested with positive and negative control sera. The result of recombinant HBsAg was similar (Table 7), but it gave good discrimination between the positive and negative controls.

Table 7. Various concentrations of conjugate.

Conc. conjugate	1:10,000	1:20,000
Synthetic Peptide		
PC	0.423	0.276
NC	0.319	0.110
Recombinant HBsAg		
PC	0.447	0.228
NC	0.129	0.072

5.4 Types of blocking reagent

For synthetic peptide, 1% HSA, 2% skimmed milk and commercial blocking reagent showed no difference absorbance between PC and NC. As for 2% skimmed milk, the absorbance of PC was higher than NC. For recombinant HBsAg, 1% HSA and 2% skimmed milk showed difference absorbance between PC and NC.

Table 8. Types of blocking reagent.

Types of blocking reagent	1% HSA	2% skimmed milk	Commercial blocking reagent
Synthetic peptide			
PC	0.203	0.336	0.160
NC	0.203	0.224	0.187
Recombinant HBsAg			
PC	0.024	0.399	nd
NC	0.007	0.066	nd

nd = not done

5.5 Variation of antigen concentration

The PolySorp polystyrene microtiter plate coated with 1.25 and 2.5 µg/ml of peptides gave lower absorbance than with 5.0 and 10 µg/ml of peptides when tested with PC and NC. No differences in the absorbance of PC and NC between 1.25 and 2.5 µg/ml were seen. For recombinant HBsAg, the polystyrene microtiter plate coated with 0.5 and 1.0 µg/ml of recombinant protein gave lower absorbance than the 1.5 µg/ml of recombinant protein when tested with PC and NC (Table 9).

Table 9. Various concentrations of antigen.

Concentration (µg/ml)	PC (O.D.)	NC (O.D.)
Synthetic peptide		
1.25	0.176	0.118
2.5	0.175	0.094
5.0	0.536	0.460
10.0	0.347	0.293
Recombinant HBsAg		
0.5	0.478	0.063
1.0	0.798	0.113
1.5	1.283	0.223

For peptide ELISA, the use of streptavidin-biotin system for increasing signal was further investigated as discribed in Chapter IV. This system gave absorbance of positive control serum higher than negative control but O.D. of negative control was too high (O.D. of positive control = 1.150, O.D. of negative control = 0.895). This system could not give good discrimination between the positive and negative controls.

In this study, the recombinant HBsAg was used as antigen for coating the ELISA plate for detection of anti-HBs antibodies. Serum samples were collected from 41 medical students and 47 normal person as described in Chapter IV. They can be divided into 3 groups; group 1 was 41 pre-vaccination samples, group 2 was 41 post-vaccination samples and group 3 is 47 persons who had been tested negative for anti-HBs. Table 10 showed that the mean of absorbance of group 1 was lower than group 2 and 3. There was significantly different absorbance of two groups of sample (pre and post-HBV vaccination) ($p < 0.0001$). Group 3 had the high absorbance value and also was not significantly different from the absorbance value of group 2 (p=0.4963).

Table 10. Mean of absorbance values from various groups.

Groups of sample	number	Mean (O.D.)±SD
1. Before HBV vaccination	41	0.741 ± 0.267
2. After HBV vaccination	41	1.029 ± 0.318
3. Anti-HBs-negative	47	0.962 ± 0.338

CHAPTER VI

DISCUSSION

1. Core promoter region and Precore region

This study showed that HBV DNA was detected in 69% (80/116 samples) of HBsAg-positive Thai patients by using PCR assay. The HBsAg-positive, but PCR-negative patients may have viral level below the detection limit of PCR or patients with certain disease e.g; hepatocellular carcinoma (HCC) do not have active viral replication. In addition, it is possible that the time of specimen collection did not coincide with the burst of viruses into blood circulation (59).

In this study, we have obtained solid information on the genotypic distribution of HBV in Thailand. There have been several data demonstrating the differences in genotypic distribution of HBV in different geographical region of the world. Some genotypes were interspreaded worldwide, whereas others were located in some restricted areas (50-52, 60). The result of the nucleotide sequences of the Thai HBV isolates described in this study and other reports showed the homogeneity of HBV genotypes found in Thailand which differed from the common genotypes found in other regions of the world. Genotype C was predominant in 80% of HBV infected Thai patients, and genotype B in 20%. The former is associated with subtype *adr* and the later with subtype *adw*. Other HBV genotypes were rarely found in Thai patients. For example, genotype A was found in only one patient who was a Western foreigner. Previous reports from Southeast Asia showed that HBV subtype *adr* was the common subtype in Thailand.

Courouce-Pauty *et al* showed subtype *adr* in 81.8% and subtype *adw2* in 18.2% of the Thai population; subtype *adr* in 87.5% subtype *adw2* in 4.2% and subtype *ayw1* in 8.3% in Laos; subtype *adr* in 42.6% subtype *adw2* in 41% subtype *ayw1* in 6.4% and subtype *ayw2* in 6.4% in Malaysia; subtype *adr* in 28.6% subtype *adw2* in 16% subtype *ayw1* in 51.2% subtype *ayw2* in 0.8% and subtype *ayr* in 3.4% in Vietnam including subtype *adr* in 20% and subtype *adw2* in 80% in Indonesia. Snitbhan *et al* reported subtype *adr* in 81% and subtype *adw* in 9% in Thai bloO.D. donors. Duanthanoem *et al* showed subtype *adr* in 81% and subtype *adw* in 19% in Thailand (32, 61, 62).

HBV infection leads to various types of liver disease. Although the mechanisms involved in the development of chronic liver disease from HBV infection are not fully clarified, it is believed that immunological mechanisms are involved in the process (63). It was shown that core antigen peptide and HBeAg peptide, which has overlapping sequence with core peptide (25, 28, 64) were recognized by cytotoxic T lymphocyte (CTL) of host (65, 66). A previous study revealed the presence of mutation clustering regions in the core region of HBV which taken from patients with chronic liver disease but not from asymptomatic carriers (67). Therefore, mutational changes of core peptide might be associated with the development of chronic liver disease as a result for immunological attack.

The transcription of the precore and pregenome mRNA, HBeAg and core protein, respectively, were reported to be regulated by the core promoter region located just upstream of the precore region (68). In this study, the change from A to T at nucleotide position 1762 (A1762T) and G to A at nucleotide position 1764 (G1764A), were detected in 13 (54.2%) of 24 HBeAg-negative, and 20 (54.0%) of 37 HBeAg-positive of the 61 Thai patients with chronic liver disease. These changes, which affect the second AT rich region, were observed most frequently in the other studies (11, 12, 54).

It is possible that these mutations are closely associated with the transcription of precore mRNA. TA changes can prevent binding of liver-enriched factor to the core promoter region. It was reported that these mutations are accompanied by reduced transcription of precore mRNA and lower expression of HBeAg (54).

From our results, the TGA mutation at position 1762 and 1764 was the most common mutation found in HBeAg-positive and HBeAg-negative individual and the high frequency of the mutation in HBeAg-positive genotype C subjects. Kidd-Ljunggren *et al* (69) reported the TGA mutation to HBeAg status for genotype A, but this was not true for genotypes B and C. Thus, other potential explanations for the frequent emergence of this mutation need to be investigated. For example, escape from proposed intracellular effects of cytokines on the core promoter may be of importance (70), as may effect on the conformation of 3´ RNA stem loop (71).

Moreover, these changes convert the amino acid sequence K130, V131 (wild type) to M130, I131 (mutant) in the region which confers a serine proteinase inhibitor function on X protein (71, 72), and which has been suggested to be important for transcriptional transactivation activity (73). An X protein carrying M130, I131 may function as well, but clearly variants with some capacity to replicate can arise, even although this region of the protein is missing entirely or both altered appreciably and truncated. Defective X gene deletion mutants depend for their survival on co-existence with virus could expressing wild type X protein.

Other mutations in the core promoter were rare. For example C1753, C1754 mutations which were always observed in conjunction with TGA at position 1762 to 1764, were associated with liver damage, whereas variation at several other positions were genotype marker. In particular, variations at nucleotide 1633 to 1638 and 1726 to 1730 were strictly associated with genotype, all genotype B strains suppose to have GGAACC and CTGAG.

Thus, analysis of these positions appear to be useful for identifying genotype B. On the other hand, nucleotide variations at T1631, A1635, C1638, C1674, C1703, C1740, T1773, C1802, G1803, G1850, A1915 and A1934 were related to genotype A. For genotype C, variations at nucleotide G1630, G1719, A1721 and G1775 were related to HBV Thai isolates.

The 'classical' precore stop mutation at codon 28 (G1896A) were recognized in 17 (70.8%) of 24 HBeAg-negative, and in 2 (5.4%) of 37 HBeAg-positive. This mutation is postulated to inhibit the production of HBeAg due to the incapability of translation of the precore message. It may be that the mutation occurred as a result of pressure from host immunological response to the virus because the reduction or cessation of the production of HBeAg, also a possible immunological target of CTL, a situation favorable for the survival of the virus.

There has been a report on the C1858 mutation in cases of the genotype B and genotype C and this was postulate to HBe serocoversion in 5 patients by preventing a precore stop mutation (G1896A). The distribution of precore mutants in the different genotypes can be explained by different stabilization energy for encapsidation signal in all the cases with M1 (C1858) or M2 (A1896) precore mutations and genotype D indicates a stabilization of the hairpin structure by the effect of these mutants (74, 75).

Thus HBe seroconversion in general implicated a reduction of (>99%) HBV DNA levels, the finding seems to indicated that the viral replication on genotype B or C is more readily reduced by immune response. This could be to due to either genotype differences in antigen expression or regulation of viral replication, but this requires further study.

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The T1653 mutation was found in only one sample from this study. There were reports on T1653 mutation which was associated with fulminant hepatitis but not with chronic liver disease (76, 77). However, this mutation reduced the levels of precore mRNA and HBeAg (78). The T1653 mutation concerts the alpha box binding site for C/EBP and related factor (79, 80) into the perfect palindromic sequence (1648TCTTATATAAGA1659) which could enhance binding affinity and this Cp/EnII activity. However, the data presented clearly indicated that this mutation exerted at least in the sequence context analyzed and in the cell line used.

The precore region mutation at nucleotide position T1856 in sample No. 49 has a proline replaced with a serine. This mutation was suggested to associate with severe disease rather than quiescent hepatitis (7). Proline residue play an important role in the secondary structure of proteins in that they terminate helices. This amino acid is near the signal peptide cleavage site (the 19th residue of precore, P19).

It is possible that the mutation results in a decrease in efficiency of cleavage and thus reduced transporting and processing of precore/core protein with relative failure of secretion. Alternatively, the secondary structure of this region may be altered, inhibit binding of the signal peptide to endoplasmic recticulum. However, it is now believed to be a naturally occurring co-circulating variant of HBV found in only Hong Kong Chinese individual (53, 81). Serine in an amino acid 15 (S15) appear to have no effect on HBe antigenicity nor secretion of HBeAg in to supernatant of recombinant vaccinia-infected cells (81).

The A1846 deletion was found in sample No. 46. It was associated with the loss of HBeAg by frameshift mutation with in the precore leader sequence. In 2 cases (No. 48 and No. 82) with deletion of 9 bp and 20 bp of the core promoter region, there has been a report that such deletions have effect to X protein function which would lead to loss 3 amino acids and frameshift (82).

Two patients (No. 91 and No. 87) had insertion of an 'A' at position 1839, leading to frameshift mutation creating stop codon at codon 33, these patients were HBeAg-negative individual. Two other patients who had 3 bp (TTG) and 2 bp (TT) insertions, one (No. 31) has an L amino acid insertion, and the other (No. 33) has frameshift in X region. These insertions are predicted to interfere with the transactivation function but a functional X protein has no effect on replication and transcription of HBV (83). These patients have HBeAg positive.

Although, these mutations occurred in HBV Thai isolates. There were at least two factors, viral factor and host factor, which were involved in HBeAg status in chronic hepatitis B patient.

Viral factor : HBeAg may play important role in the high rate of development of chronic hepatitis B infection in children born to mothers who are HBeAg positive (3, 84, 85). It has been shown that HBeAg crosses the human placenta (86) and demonstrated that *in utero* exposure to HBeAg leaded to significant period of neonatal T-cell tolerance to HBeAg and HBcAg in nontransgenic offspring of HBeAg expression transgenic female mice (87). Thus it may be postulated that HBeAg reduced the cytotoxic T cell response to infected hepatocytes (87,88).

A mutation in precore region of nucleocapsid reading frame creates a termination codon that prevents translation of HBeAg. This appears to be a successful escape mutation that emerges under the selection pressure of anti-HBe antibody. However, it has been established that virus bearing the precore mutation may be found in patients in whom HBV replication has been controlled (5). DNA sequencing of viral isolates from patients who seroconverted from HBeAg-positive to anti-HBe-positive revealed that mutations in antigenic determinants of the core region are associated with persistent viremia, indicating that the precore mutation alone is probable insufficient for successful evasion of the immune response.

Host factor: Major histocompatibility complex (MHC) is one of the risk factors of chronic hepatitis B infection. Milich *et al* (89) showed the potential of the nucleocapsid antigens (HBcAg and HBeAg) of HBV to preferentially elicit either a Th1 or a Th2 dominant response in B10.s and B10 mice. These mice were immunized with HBcAg and HBeAg and developed Ag-specific T cell proliferative response. B10.s mice preferentially developed a Th1-like response, whereas B10 mice preferentially developed a Th2-like response. Predominance of HBeAg-specific Th2-like cell were associated with HBV persistence (90,91).

Antibody to nucleocapsid antigen, the role of the antibodies response to the HBV nucleocapsid antigens (HBcAg and HBeAg) in HBV pathogenesis is not clear. It is generally accepted that they do not neutralize viral infectivity because they were present in the high titers not only during acute hepatitis phase but also in patients with chronic HBV infection. However, the passively administered anti-HBe antibodies appear to protect chimpanzees against HBV infection (92), suggesting that they play some obscure role in HBV neutralization.

Because the T cell response to HBcAg and HBeAg is strong during acute hepatitis and weak in chronically infected patients, the strong antibody response to HBcAg in chronically infected patients may indicate in part to the fact that it can functions as both a T cell-independent and T cell-dependent antigen (93).

2. Nucleotide Sequencing of *S* Gene

Four positions of amino acids (126,131,134 and 143) in 'a' determinant were different from *adr* prototype, most of subtype *adr* amino acid 126 is isoleucine (I126), but the majority of the Thai isolates had threonine (T126) at this position. Threonine is an amino acid with uncharged polar side chain, whereas isoleucine is an amino acid with non polar side chain. The side chains of these non polar amino acid tend to cluster together in the interior of the protein. This phenomenon is due to the hydrophobicity of the non polar R groups. HBsAg particles containing T126 have an antigenicity different from that of those containing I126, and the reactivity with monoclonal difference in that they had T126 in place of I126 (94).

At position 131, asparagine (N131) and threonine (T131) are amino acids with unchanged polar side chains. Side chain of Thr contain a polar hydroxy group whereas side chain of Asn contain amide group. This position is the variable amino acid in *d/y* or *w/r* specificity (33, 95, 96) but position 131 did not appear to be involved since substitution of Asn at this position for Ala (A131) had no effect on anti-d binding (97).

At position 134, phenylalanine (F134) is a non polar side chain amino acid, but tyrosine (Y134) is a polar side chain amino acid. Phenylalanine and tyrosine are involved in *d/y* specificity, however two Thai isolates (No. 607 and 652) were of subtype *adr* in genotype C (33, 95, 96) .

At position 143, there is a threonine (T143) in groups A and B and a serine (S143) in groups C to F genome. These amino acids have zero net charge at neutral pH. However, the pepscan approach showed that the replacement of residue 143 resulted in significant reduction in binding (98).

For other positions in 'a' determinant, the nucleotide sequence of Thai isolates were similar to that prototype isolate (m38454).

3. ELISA for Detection of Anti-HBs antibody

In this study, we failed to establish an ELISA system for the detection of anti-HBs antibodies by using either synthetic peptide or recombinant HBsAg.

The affinity of anti-HBs positive human serum for linear peptide (aa 139-147) is higher than for the peptide from aa 124 to 137. If the former peptide is cyclized between the terminal cysteines, the affinity was clear (99). The region particularly between aa 139 and 147 is highly conserved. There have been a report that this region indicated only the second loop is likely to be real and that the first loop is incorporated within a larger loop from aa 107 to 138 by using phage epitope libraries (100). Previously study of peptide of aa 124-147 reported the auto-assemble of the peptide to form pentameric molecule (101). The multimer form was important for 'a' determinant of HBsAg. Our peptide had 3 aa addition at N terminal of aa 124-147 peptide that will form loop structure (102). However, this could not be proved because it was not able to demonstrate the auto-assemble of the peptide used in this study. In order to prove this hypothesis, the synthetic peptides should be bound directly onto a microtiter plate using two buffers with different pH 7.2 and 5.6 for binding to solid phase. The method does not work equally well for the peptide and loss of peptides during the ELISA procedure can not be excluded (103).

Each step in the ELISA system was optimized step by step from types of microtiter plate, concentrations of peptide or recombinant HBsAg, dilutions of serum sample, types of blocking buffer, concentrations of conjugate and amplified O.D. signal by using biotin-streptavidin system which will discuss as follow.

Microtiter plate types, O.D. signal of negative control was higher or nearly the same in MaxiSorp and Immulon II when testing with peptide and recombinant HBsAg coated plates as shown in Table 5. These two types of microtiter plates were modified by its manufacture for increasing affinity to molecules with mixed hydrophilic and hydrophobic domain (104). The different O.D. signal of positive and negative control sera could be obtained from PolySorp which less polar and has affinity with more hydrophobic molecules. When looking at the peptide which was 27 amino acids long, only 8 were hydrophobic amino acids. We expected that these 8 hydrophobic amino acids should bind to surface and shoot up the other 19 hydrophilic amino acids to be epitope domain to react with specific anti-HBs antibodies. The binding of the peptide to MaxiSorp and Immulon II may not be as expected, that mean hydrophilic part may bind to the surface and leave the hydrophobic part to make non-specific signal of negative control serum. Moreover, physical adsorption of antigens (peptide and recombinant protein) onto a surface microtiter plate involved the risk of hiding of active site due to distortion of molecules (105).

In general, the binding of protein to polystyrene surface is achieved through hydrophobic interactions. Proteins have their most hydrophilic residues facing outside, whereas the more hydrophobic residues are oriented towards the inside (106). A previous report discribed a study using similar peptide, but with 3 amino acids (CKT) shorter and two different amino acids (T131N and S143T) in ELISA used the polystyrene microtiter plate. However the absorbance value of negative control was not given.

Variation of antigen concentration, when the PolySorp plate was coated with various concentrations of peptide as 1.25, 2.5, 5.0 and 10.0 µg/ml , the maximum O.D. of both positive and negative control sera were obtained from 5.0 µg/ml peptide coated well. The lower O.D. in 10 µg/ml peptide coated well than 5.0 µg/ml peptide coated well may resulted from a high binding density of the peptide that can effect on the conformation of antigen (107) and the rate of antigen desorption during ELISA process (108). However, we could not use 5 µg/ml peptide concentration for coating plate since the O.D. of negative control serum was too high. So the optimal peptide concentration in this experiment was 1.25 µg/ml or 1.0 µg/ml that is generally used concentration. By using this condition as peptide ELISA, excepted the recombinant protein concentration of 0.5 mg/ml .

Dilutions of serum. For recombinant HBsAg, in order to increase O.D. signal of positive control serum and decrease O.D. signal of negative control serum, various dilutions of serum were tested. The result showed that the 1:10 dilution gave highest O.D. of positive control sera in both peptide and recombinant HBsAg coated well. But at this concentration, the negative control serum also gave the highest O.D. So the optimal dilution of serum was 1:100 which gave good. discrimination between positive and negative controls. For peptide, they gave low O.D. both of positive and negative control sera. The limited number of peptide-binding antibodies competed with the recombinant HBsAg may effect in this ELISA procedure (109).

After each steps was optimized, The ELISA system using recombinant HBsAg was evaluated with clinical serum samples. However, the O.D. of negative samples were too high, as well as those for the 41 pre-vaccination and 47 anti-HBs negative sera. These are non-specific binding in normal healthy. One reason that could be possible was the impurity of our recombinant HBsAg. Since, this recombinant HBsAg was yeast-derived, contamination of some yeast protein may interfere the ELISA system.

The effect of yeast contaminated protein can account on both positive and negative controls. Unfortunately, we have obtained only limited amount of recombinant protein and could not evaluate this system further.

CHAPTER VII

CONCLUSION

The genotypes of HBV isolated from Thai patients were determined in this study. Of the 61 patients, one was infected with HBV genotype A, 18 with genotype B and 42 with genotype C, with the frequency of 1.6 %, 29.5% and 68.9%, respectively. Genotype-specific nucleotides were observed.

The role of core promoter sequence changes in the apparent loss of HBeAg circulation was evaluated, we found that mutations at position 1762 and 1764 (T, A) were detected in 33 (54.1%) out of 61 patients. Of these, 20 (60.6%) patients were HBeAg-positive, 13 (39.4%) patients were HBeAg-negative. Whereas wild type (A, G) were present in 26 (42.6%) out of 61 patients. Of these, 17 (65.4%) patients were HBeAg-positive, 9 patients (34.6%) were HBeAg-negative. No significant differences were observed between these groups. In addition, the mutations in the precore region were found; such as a nucleotide substitution C to T at position 1856 (C1856T), T1858C, G1896A, G1898A and G1899A. At position 1896, G1896A mutation was found in 19 (31.1%) out of 61 patients. Two of nineteen (10.5%) were HBeAg-positive and 17 of 19 (89.5%) were HBeAg-negative. G1896r, mixed infection with both wild type and mutant viruses were found in 7 (11.5%) patients and all but one were HBeAg-positive.

Nucleotide insertions were found in 4 isolates and nucleotide deletions were found in 3 isolates, 6.5% and 4.9% respectively.

In *S* gene, the overall similarity of nucleotides sequence (93.0-99.0%) and amino acid sequence (88.9-99.6%) were found when compared with each other. Four positions (126, 131, 134 and 143) in 'a' determinant of *S* gene were different from *adr* prototype. They are involved in *d/y* and *w/r* specificity.

Unfortunately, we could not set up the detection system for anti-HBs antibody by ELISA.

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APPENDIX

REAGENTS FOR PCR AMPLIFICATION

10x PCR buffer

(containing 500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$)

2.5 M KCl	2.00 ml
1M Tris-Cl pH 8.3	1.00 ml
1M $MgCl_2$ (Merck)	0.15 ml
DEPC- treated water to	10.00 ml

The solution was kept in 1 ml aliquot at -20°C.

dNTP

(containing 2.5 mM of each dATP, dGTP, dCTP, dTTP (Promega) in DEPC-treated water)

100 mM dATP (Promega)	20.00 µl
100 mM dGTP (Promega)	20.00 µl
100 mM dCTP (Promega)	20.00 µl
100 mM dTTP (Promega)	20.00 µl
DEPC-treated water	720.0 µl

The solution was kept in 50 µl aliqout at -20°C.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

6x DNA loading buffer

Bromophenol blue	0.025	gm
Glycerol	3.00	ml

Final volume was adjusted to 10 ml with sterile distilled water. The buffer was aliquoted into 1.5 ml vials and stored at 4°C.

2% agarose gel (w/v)

Agarose gel (Gibco)	2.00	gm
1x TBE buffer	100.0	ml

Melted in microwave oven until completely dissolved.

1x TBE buffer

Tris base (Sigma)	10.80	gm
Boric acid (Fluka)	5.50	gm
0.5 M EDTA (pH 8.0)	4.00	ml

Dissolve and adjust the volume to 1,000 ml with distilled water.

Ethidium bromide (10 mg/ml)

Ethidium bromide (Sigma)	1.00	gm
Distilled water	100.0	ml

The solution was stored in a dark bottle at 4°C.

REAGENT FOR ELISA

0.05 M Carbonate buffer pH 9.6

Sodium carbonate (Na_2CO_3) (Merck)	1.70	gm
Sodium bicarbonate ($NaHCO_3$) (Merck)	2.86	gm
Distilled water to	1000	ml

Adjust to pH 9.6 by HCl or NaOH.

0.1 M acetate buffer pH 5.6

Solution A: 0.2 M acetic acid

Glacial acetic acid (CH_3COOH) (Merck)	11.55	ml
Distilled water to	1000	ml

Solution B: 0.2 sodium acetate

Sodium acetate ($C_2H_3O_2Na$) (Merck)	16.20	gm
Distilled water to	1000	ml

Mixed 4.8 ml volumes of solution A and 45.2 ml volumes of solution B and added distilled water to final volume of 100 ml.

2% skimmed milk in 1x PBS

Skimmed milk (Marvell)	0.50	gm
1x PBS	25.00	ml

Dissolved completely and prepared before use.

Streptavidin-peroxidase solution

Peroxidase-labeled streptavidin was purchased from Kirkegaard&Perry Laboratory (Maryland, USA). The reagent was dissolved in 50% glycerol and kept in 20 µl aliqote at -20°C. The conjugate was 1:2,000 fold diluted with 2% skimmed milk in 1x PBS before use.

0.15 M Phosphate buffered saline (PBS) pH 7.2

NaCl	8.00	gm
KCl	0.20	gm
Na_2HPO_4	1.44	gm
KH_2PO_4	0.24	gm

Dissolved in distilled water, adjust to pH 7.2 with HCl.

Distilled water to	1,000	ml

Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches and stored at room temperature.

2 N H_2SO_4

Distilled water	454.0	ml
Concentrate sufulic acid (95-97%H_2SO_4)	46.0	ml

0.02 M Citrate phosphate buffer pH 5.0

Solution A: 0.1 M citric acid

Citric acid ($C_6H_8O_7.H_2O$) (Merck)	21.01	gm
Distilled water to	1000	ml

Solution B: 0.2 M dibasic sodium phosphate

Sodium phosphate dibasic, anhydrous	28.40	gm
Distilled water to	1000	ml

Mixed 243 ml of solution A and 257 ml of solution B and then added distilled water 400 ml.

Adjust pH to pH 5.0 by adding solution A or B, and then bring final volume to 1000 ml

GENERAL REAGENTS

1M Tris-Cl pH 8.0

Trisma base (Sigma)	12.11	gm

Dissolved in distilled water and adjusted pH to 8.0 with HCl

Distilled water to	100.0	ml

Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches and stored at room temperature.

0.5 M EDTA pH 8.0

EDTA (Amresco)	18.60	gm.

Dissolved in distilled water and adjusted pH to 8.8 with NaOH

Distilled water to	100.0	ml

Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches and stored at room temperature.

1 M NaOH

NaOH (Fluka)	4.00	gm
Distilled water to	100.0	ml

Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches and stored at room temperature.

1 M $MgCl_2$

$MgCl_2$ (Merck)	20.30	gm
Distilled water to	100.0	ml

Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches and stored at room temperature.

0.1% DEPC-treated water

Distilled water	999.0	ml
Diethylpyrocarbonate (Fluka)	1.00	ml

The solution was vigorously mixed, incubated at 37°C overnight, autoclaving for 15 minutes at 121°C, 15 lb/square inches and stored in a DEPC-treated bottle at room temperature.

Chloroform:Isoamyl alcohol (24:1)

Chloroform (Merck)	24	volumes
Isoamyl alcohol (Merck)	1	volume

Mixed them together and stored in a DEPC-treated dark bottle at room temperature.

2 M Sodium acetate pH 4.0

Sodiun acetate (Merck)	16.40	gm

Dissolved in distilled water and adjusted pH to 4.0 with glacial acetic acid

Distilled water to	100.0	ml

The solution was mixed, filtered through 0.45 mm fiter and stored in a DEPC-treated bottle at room temperature.

Phenol:chloroform:deionized water (68:14:18)

Phenol (Merck)	68	volumes
Chloroform (Merck)	14	volumes
Deionized water	18	volumes

Mixed them together and stored in a DEPC-treated dark bottle at room temperature.

95% ethanol

Absolute ethanol (Merck)	95.0	ml
Deionized water	5.0	ml

The solution was mixed, filtered through 0.45 mm fiter and stored at room temperature.

70% ethanol

Absolute ethanol (Merck)	70.0	ml
Deionized water	30.0	ml

The solution was mixed, filtered through 0.45 mm fiter and stored at room temperature.

BIOGRAPHY

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INSTITUTION ATTENDED	Chiangmai University, 1983-1987:
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