NUCLEIC ACID SEQUENCE ANALYSIS OF THE DISTAL X GENE REGION CONTAINING THE BASIC CORE PROMOTER OF THE HEPATITIS B VIRUS IN SOUTHERN AFRICAN ASYMPTOMATIC CARRIERS OF THE VIRUS AND HEPATOCELLULAR CARCINOMA PATIENTS

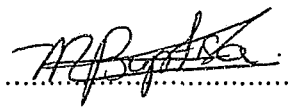
Marina Da Conceição Pinto Azevedo Baptista

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2000

Declaration

I, Marina Da Conceição Pinto Azevedo Baptista declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



17th day of November, 2000

In loving memory of my husband

Manuel Jorge Dinis Baptista

1967 – 1998

"You will forever more live in my heart Jitinha.
May you have eternal rest."

Publications and presentations arising from this study

Baptista, M., Kramvis, A. & Kew, M.C. High prevalence of $1762^{T}1764^{A}$ mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology, vol. 29, no. 3, pp. 946-953, 1999.

Azevedo, M., Kew, M.C. & Kramvis, A. Sequence analysis of the basic core promoter of hepatitis B virus from sera of southern African black asymptomatic carriers and hepatocellular carcinoma. Cancer '97. Second National Conference; 1997 Feb 12-14; Langerbaan. Abstract number: L8.

Abstract

The purpose of this study was to identify mutations in the basic core promoter and enhancer II region of the hepatitis B virus (HBV) that might result in the hepatitis B virus e antigen (HBeAg)-negative phenotype and contribute to hepatocarcinogenesis in black African carriers of the virus. The basic core promoter/enhancer II overlaps the X gene. HBV DNA from serum of 47 asymptomatic carriers and 50 patients with hepatocellular carcinoma and from 29 tumorous and 10 nontumorous liver tissues was amplified and sequenced directly. That part of the enhancer II region not overlapping the basic core promoter was reasonably well conserved in all samples. Missense mutations at positions 1809 and 1812 were found in 80% of all sequences and may represent wildtype sequence in southern African isolates. Nucleotide and amino acid divergences were higher in the basic core promoter of hepatocellular carcinoma patients than of asymptomatic carriers ($p<0.0001$). This applied particularly to the paired 1762 adenine to thymine (1762^{T}) and 1764 guanine to adenine (1764^{A}) missense mutations, the prevalence of which was 66% in patients with hepatocellular carcinoma compared with 11% in asymptomatic carriers ($p<0.0001$). There was no association between the presence of $1762^{T}1764^{A}$ and the rate of HBeAg negativity, although these mutations suppressed HBeAg titres in HBeAg-positive patients. Suppression of HBeAg expression as well as alteration of amino acid sequence of the X protein may be contributing factors in the development of hepatocarcinogenesis.

Acknowledgements

I would like to thank:

- My supervisors, Professor Kew and Dr Kramvis, for all their advice and guidance, and for having had faith in me when I lacked it. Anna, I can never thank you enough for all your support. I believe that your kind words and genuine concern for my well being have kept me sane and given me strength to carry-on when all seemed lost.
- All my friends and colleagues at the Molecular Hepatology Research Unit and Department of Medicine. Thank-you for putting up with my impatience and mood-swings.
- My brother Sergio. Thank-you for all your support with this dissertation and for always being there for me. You have been my confidant and rock.

I gratefully acknowledge the financial support that I have received from the University of the Witwatersrand, Foundation for Research and Development (FRD), and The Poliomyelitis Research Foundation. I'm also grateful for the grants received from the H.E. Griffin Cancer Trust and Medical Faculty Research Endowment Fund that supported this project.

TABLE OF CONTENTS

List of Figures

List of Tables

Abbreviations

aa:	amino acid
ASC:	asymptomatic carrier
BCP:	*basic/basal core promoter*
bp:	base pair
CURS:	core upstream regulatory sequence
dd:	dideoxyl
DNA:	deoxyribonucleic acid
dNTP:	deoxyribonucleotide triphosphate
DR:	direct repeat
HBc:	hepatitis B core
HBcAg:	hepatitis B core antigen
HBe:	hepatitis B e
HBeAg:	hepatitis B e antigen
HBsAg:	hepatitis B surface antigen
HBX:	hepatitis B X
HBXAg:	hepatitis B X antigen
HBV:	hepatitis B virus
HCC:	hepatocellular carcinoma
mRNA:	messenger ribonucleic acid
NRE:	negative regulatory element
nt:	nucleotide
ORF:	open reading frame
PCR:	polymerase chain reaction
RNA:	ribonucleic acid
rpm:	rotations per minute
SDS:	sodium dodecyl sulfate
TBP:	TATA-binding protein
URR:	upstream regulatory region

Chapter 1: INTRODUCTION

Although virus B hepatitis is an important public health problem worldwide, it is of particular concern in sub-Saharan Africa and the Asian Pacific countries, where HBV is endemic. HBV carriers are known to be long-term reservoirs of the virus and they frequently develop chronic disease, including hepatocellular carcinoma. The mechanisms behind the various clinical manifestations of hepatitis B infection are still poorly understood.

Traditionally, the various clinical manifestations were attributed to variation in the host's immune response(s). However, recent studies have taken into account the possible role for HBV mutants. This chapter gives a brief overview of HBV, concentrating particularly on the core promoter, which was the region studied.

1.1 HEPATITIS B VIRUS

1.1.1 History

Although an acute form of hepatitis was noted more than 100 years ago, the identification and characterization of HBV was a slow and difficult process (Fields *et al.*, 1985). In 1947 MacCallum introduced the term "hepatitis B" to characterize this infection, and this name was subsequently adopted by the World Health Organization Committee on Viral Hepatitis. In 1963 Blumberg discovered a previously unknown protein in the blood and termed it the Australian (Au) antigen. Five years later Prince, Okachi and Murakami found that the Au antigen (today known as the hepatitis B virus

surface antigen (HBsAg)) was found only in serum of patients with hepatitis B. In 1973 Dane identified the hepatitis B viral particles in the serum of HBV infected patients (Fields *et al.*, 1985).

With the discovery of the HBsAg and viral particles, rapid advances were made in understanding the epidemiology of the virus, its course of infection, the recognition of associated diseases, and the physical nature of the virus.

1.1.2 Epidemiology

Hepadnaviruses have been found in humans and non-human primates. Humans remain the principal reservoir of HBV. HBV is globally distributed, its prevalence, as measured by the presence of HBsAg, varying in different regions, (figure 1), (Robert, 1998). Clinically the HBsAg is used as a marker to indicate HBV infection because it is the first antigen produced after infection, and it is also the last antigen to disappear following recovery.

The incidence of HBV is lowest (<0.5%) in developed countries (e.g. Great Britain, Canada, United States, Scandinavia, and some European countries), where the living standards are high. Conversely, in developing countries (e.g. China, Africa, Oceania, South Pacific, Middle East and some parts of South America), HBV incidences are high (5 – 20%), (IARC monographs, 1994).

In countries with low incidences of HBV, the disease is most commonly seen in teenagers and young adults. These include certain groups who are sexually promiscuous or who have frequent contact with blood or blood products. However, in

countries heavily affected by HBV, it is mostly infants and young children who are affected. They acquire their infection either peri-natally (mother-to-child transmission) or horizontally from close childhood interactions (IARC monographs, 1994).

Within any one country, the HBsAg carrier rate may vary amongst different ethnic groups. In South Africa more than 10% of blacks (i.e. 3.3 million) are chronic carriers of HBV, whereas less than 1% of South African Caucasions are carriers of the virus (Kew *et al.*, 1975; 1976).

1.1.3 Pathogenesis of HBV infection

HBV infection can lead to a number of clinical consequences that are influenced by age, sex and the state of the immune system (Feitelson, 1994).

In adults, an acute exposure to HBV either results in a transient subclinical infection or in a short episode of typical acute hepatitis. In both cases the majority of patients recover from the infection. Rarely, patients who develop acute liver disease rapidly progress to fulminant hepatitis, which has a rapid onset and is associated with a high mortality, (figure 2), (Feitelson, 1994). Five to 10% of patients exposed to HBV (either with subclinical disease or acute hepatitis) develop a chronic infection, characterized by the persistence of HBsAg and/or virus in the blood for more than 6 months. Chronic HBV infection may either be symptomatic or asymptomatic. In either case, chronic HBV infection may lead to cirrhosis, dysplasia and hepatocellular carcinoma (HCC), (figure 2), (Feitelson, 1994).

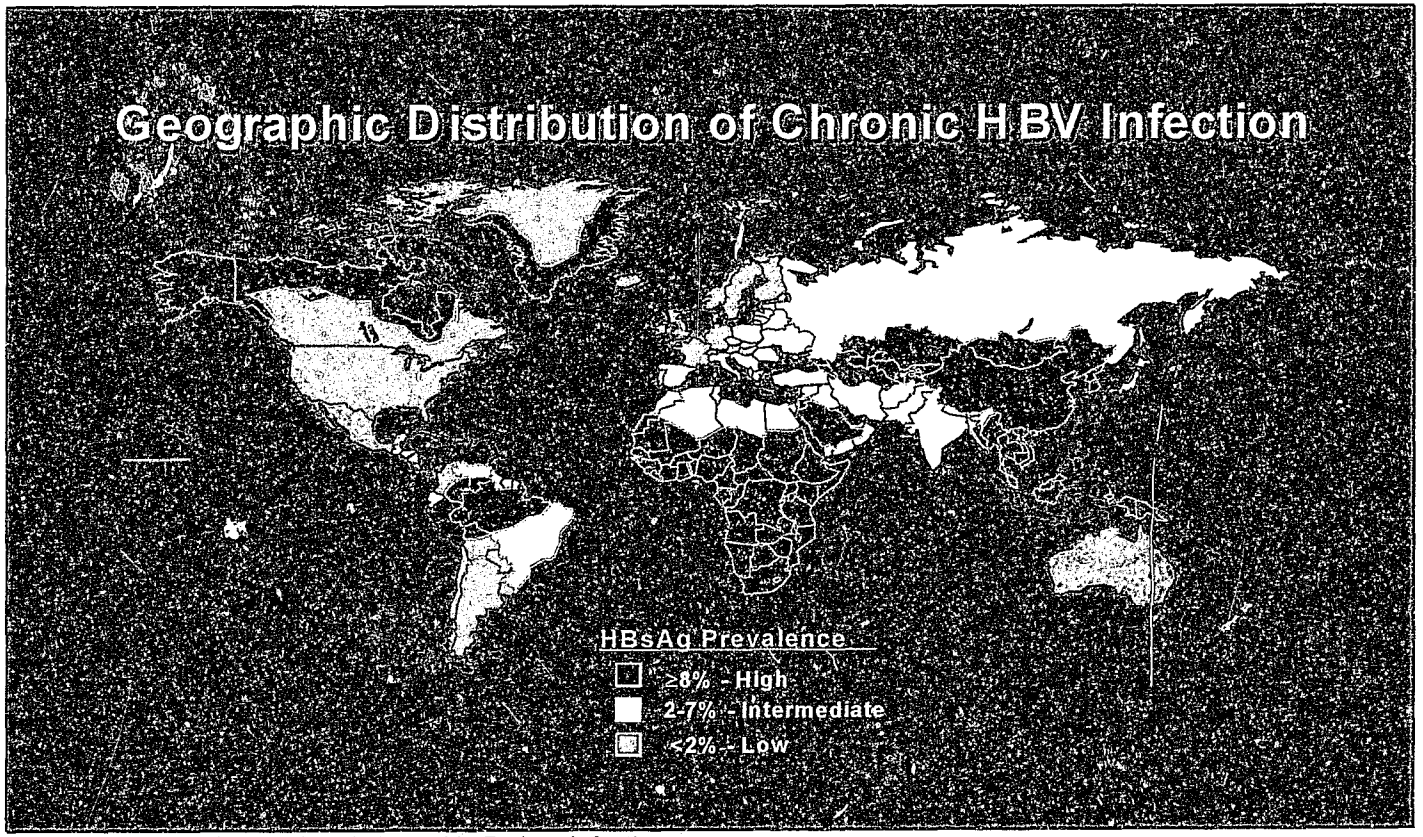


Figure 1 Global distribution of chronic hepatitis B virus infection.

(Robert, 1998)

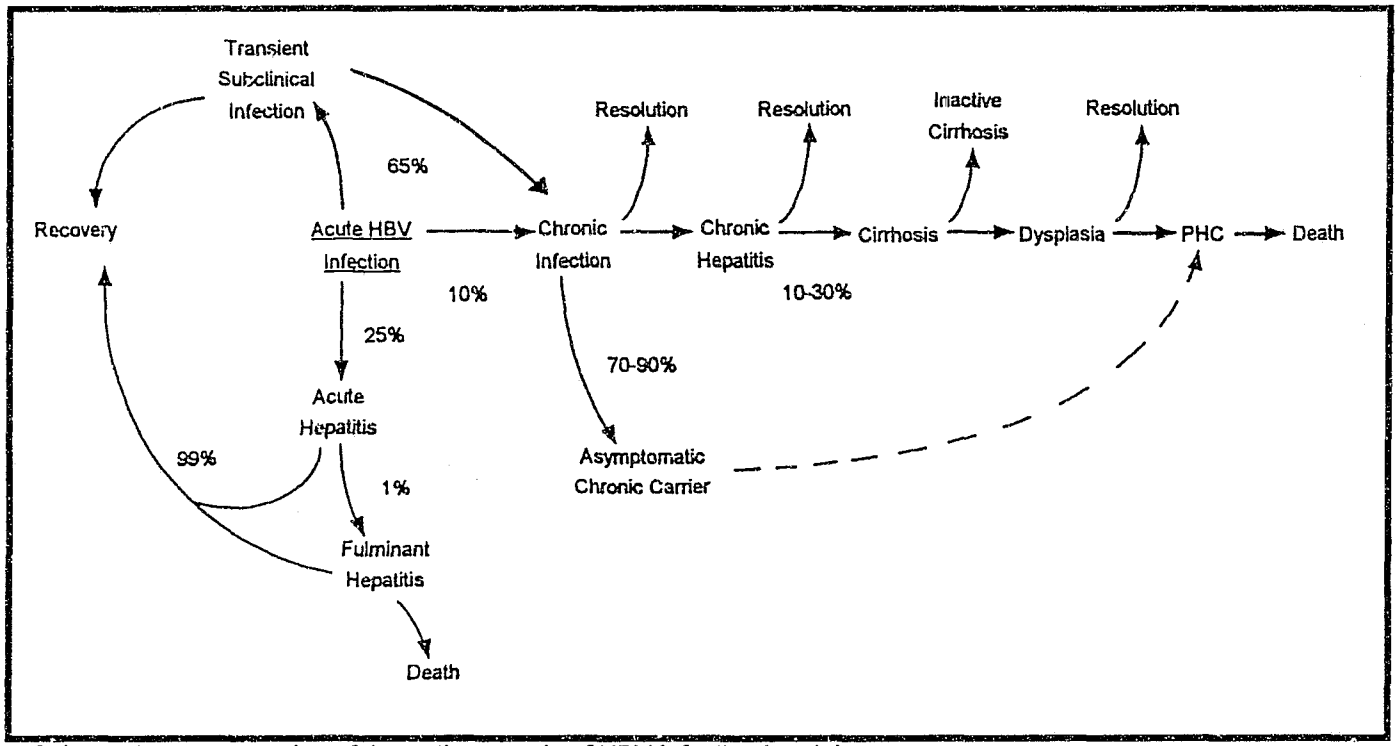


Figure 2 Schematic representation of the pathogenesis of HBV infection in adults.

(modified from Feitelson, 1994)

The course of perinatal infections differs from that in adults. Infants born to mothers who are chronic carriers of HBV are very likely to become infected during or shortly after delivery, and 80-90% become chronic carriers of the virus. These infants are at an increased risk of dying from cirrhosis or HCC (Feitelson, 1994).

1.1.4 Virion structure

HBV belongs to a family of viruses known as the *Hepadnaviridae*. It is a hepatotropic DNA virus (Gerber *et al.*, 1985).

1.1.4.1 Ultrastructure and physical properties

Electron microscopy of partially purified preparations of HBV from human serum revealed 3 types of particles: 1) variable length filaments, 2) spheres (heterogeneous in size and appearance) and 3) double-shelled particles, (figure 3(a)), (Fields *et al.*, 1985; Ganem *et al.*, 1987).

The ~22nm spheres and filaments were found to be noninfectious, nucleic acid-free defective viral particles, made-up of excess viral coat protein (HBsAg). They accumulate to high levels in the blood of infected patients and are thought to act as "dummy" particles that serve to adsorb neutralizing antisurface antibodies during the progression of infection, (figure 3(b)), (Fields *et al.*, 1985; Gerber *et al.*, 1985; Ganem *et al.*, 1987).

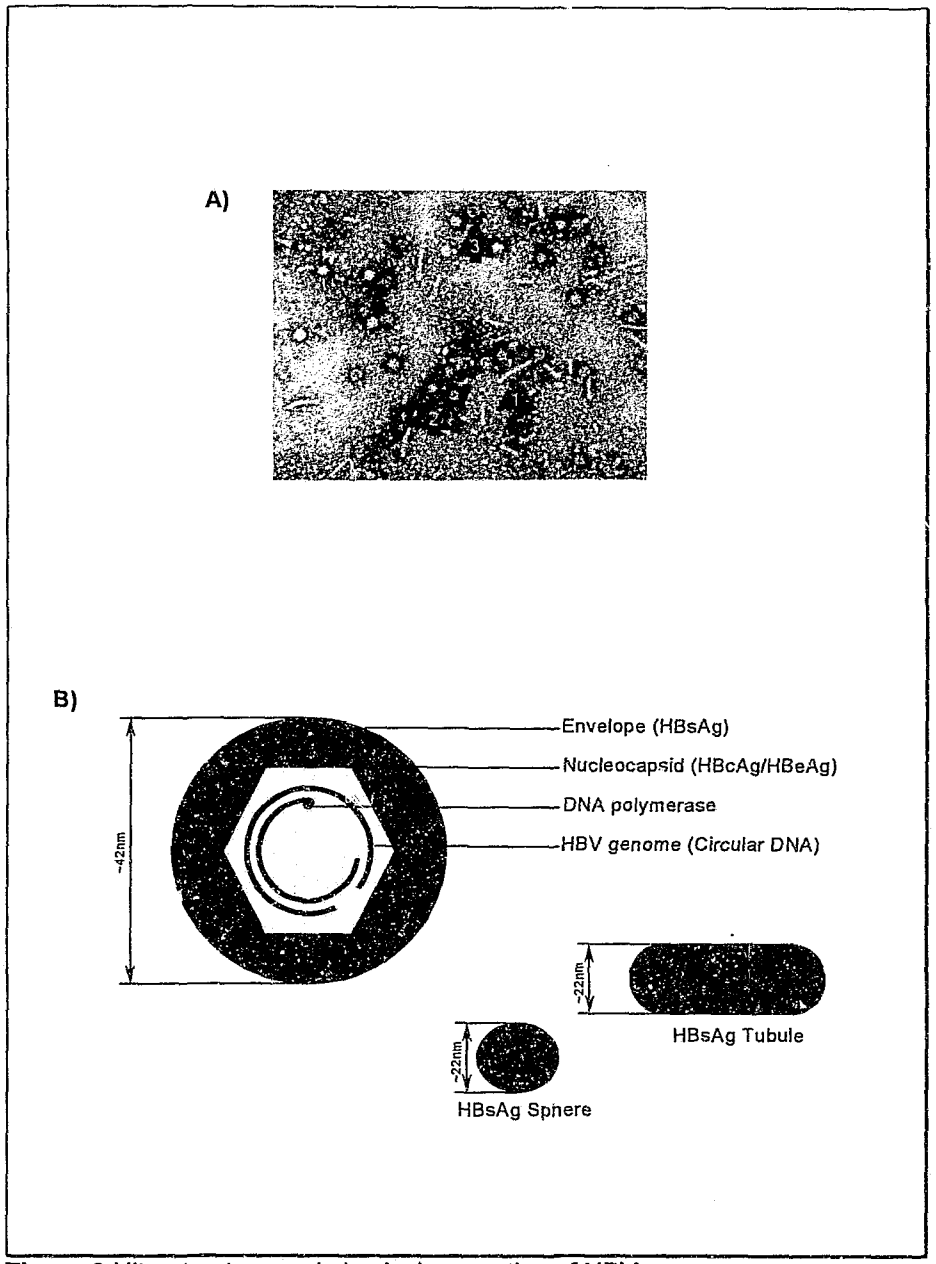


Figure 3 Ultrastructure and physical properties of HBV.

A) Three types of particles in the serum of a patient with chronic active hepatitis B: 22nm filaments and spheres (arrow 1 and 2, respectively) and 42nm hepatitis B virions or Dane particles (arrow 3), (Belshe *et al.*, 1984).

B) Schematic representation of the structure and components of the HBV particles (Ganem *et al.*, 1987).

The intact, infectious 42-47nm spherical-shaped HBV virions or Dane particles are found less frequently than the spheres and filaments in blood of infected individuals. The outer envelope of the virion is ~7nm in thickness and is mainly made-up of HBsAg. Upon removal of the outer envelope, a 22-25nm diameter nucleocapsid or core is revealed. The nucleocapsid contains the hepatitis B virus core antigen (HBcAg) and HBeAg. Within the nucleocapsid is housed the viral DNA with a covalently attached polypeptide and at least 2 enzymes: DNA polymerase and protein kinase, which phosphorylates the major structural polypeptide of the virion core, (figure 3 (b)), (Gerber *et al.*, 1985; Ganem *et al.*, 1987).

1.1.4.2 Genome structure

HBV is the smallest known mammalian DNA virus, with a genome of 3182-3221 nucleotides, depending on the genotype. Its genome is circular and partially double-stranded, (figure 4), (Gerber *et al.*, 1985; Tiollais *et al.*, 1985, 1988). The short, incomplete, plus strand is of variable length, ranging from 50-100% of the length of the long (minus) strand. The 3'end of this strand, which is variably located, is complexed with DNA polymerase, (figure 4). Under appropriate conditions, the short strand can be elongated to form a double-stranded DNA molecule for virus replication. The 5'end of the short strand has a fixed location and an oligoribonucleotide attached to it, which acts as a primer for DNA synthesis (Gerber *et al.*, 1985; Ganem *et al.*, 1987).

The complete (minus) strand has a fixed length of ~3200 nucleotides. It is the template for the synthesis of all viral messenger RNA (mRNA) transcripts and bears the 4 overlapping open reading frames (ORF) that encode for the surface, polymerase, X, and precore/core genes, (figure 4), (Gerber *et al.*, 1985; Tiollais *et al.*, 1985,1988).

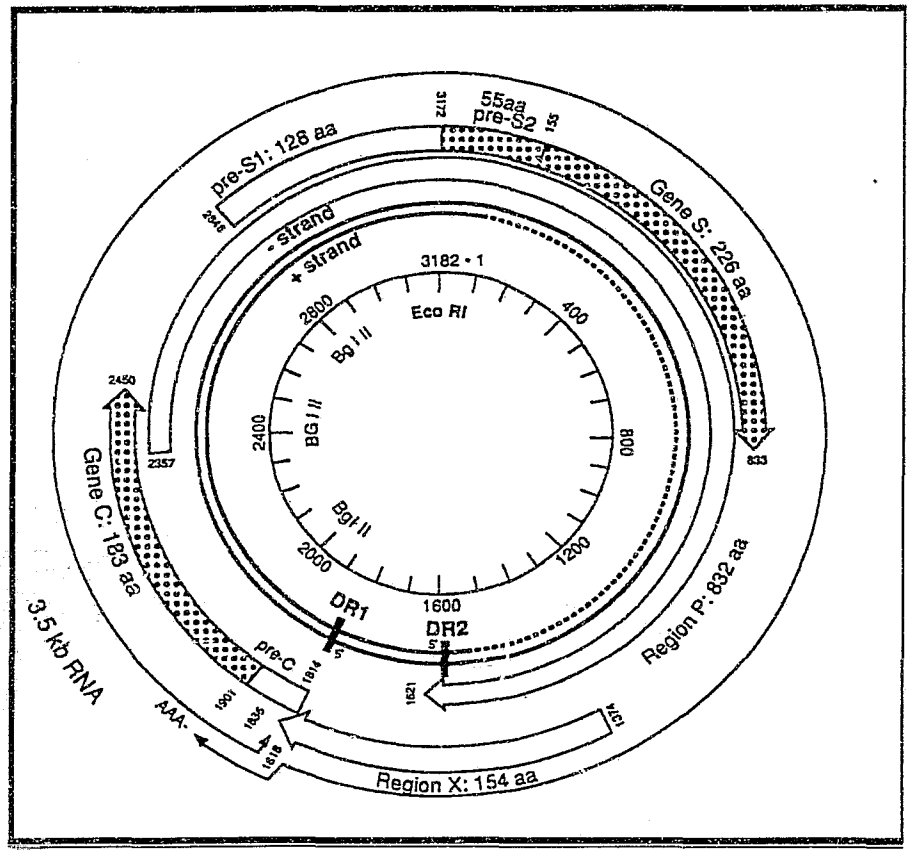


Figure 4 Physical and genetic map of HBV DNA.

The 5'end of the minus strand is base-paired with the 5'end of the plus strand. The dashed line corresponds to the variable single-strand region. The single *Eco*R1 site is used as the point of origin in the physical map. The broad arrows surrounding the genome correspond to the four large open region on the minus strand transcript. These four coding regions are called S (divided into pre-S1, pre-S2 and gene S), P, X, and C. The number of amino acids alongside each region corresponds to the length of the corresponding polypeptide. The 3.5kb pregenomic RNA template is also shown (Tiollais *et al.*, 1985).

1.1.4.2.1 Surface gene

The surface or envelope ORF encodes for 3 surface proteins. It is able to do this because it has three in-frame AUG start sites, all sharing a common terminal codon (UAG/UAA). The proteins encoded for are the small (S), middle (M) and large (L) surface antigens (Ganem *et al.*, 1987; Feitelson, 1994).

The SHBsAg is 226 amino acids (aa) long. It is produced in large quantities by the virus and is the major constituent of the envelope of the Dane and subviral particles. It is highly antigenic, containing an epitope that allows for the subtyping of HBV (Ganem *et al.*, 1987; Feitelson, 1994).

The MHBsAg comprises the S region and the upstream pre-S2 region. The pre-S2 region is a 55aa domain, thus resulting in a MHBsAg of 281aa. MHBsAg, like SHBsAg is found in Dane and subviral particles. This antigen is thought to indirectly aid with viral attachment to cells (Ganem *et al.*, 1987; Feitelson, 1994).

The LHBsAg, the largest of the surface antigens, contains pre-S1 along with pre-S2 and the S domains, resulting in a total length of 409aa. This antigen is primarily found in Dane particles. It is thought to be involved in host cell attachment (Ganem *et al.*, 1987; Feitelson, 1994).

1.1.4.2.2 Polymerase gene

The polymerase ORF is the largest ORF, and it overlaps all the other ORFs. It appears to have 4 distinct domains (Radziwill *et al.*, 1990). The first is the amino-terminal domain, which encodes a protein that is linked to the 5'end of the viral DNA minus strand. This protein has been termed "primase" and has been found to be vital for priming minus strand synthesis. The second domain appears to have no specific function and may be a spacer. The third domain encodes for the DNA-dependent polymerase and reverse transcriptase. The fourth domain has RNAse H activity that degrades the RNA pregenome during DNA synthesis (Preisler-Adams *et al.*, 1993).

1.1.4.2.3 X gene

The X ORF encodes for a 145-154aa cytosolic hepatitis B X protein (HBX) that has a fairly complex structure. Its secondary structure consists of 4 alpha-helix or coil regions, 4 beta-sheet regions and 4 turning regions (figure 5(a)). Maintenance of this secondary structure is thought to be essential for a conformationally active form of HBX (Kim *et al.*, 1993).

Amino acid analysis of HBX shows: 1 proline/serine rich region (aa29-46), 2 basic regions (aa72-98 and aa128-140), 1 acidic region (aa106-127) and 1 leucine zipper-like region, containing at least six leucine residues with four to seven aa intervals (aa105-143), (figure 5(b)), (Kim *et al.*, 1993).

Mutation analysis of the HBX protein has revealed that it consists of a number of essential and non-essential regions (figure 5(c)). The amino-terminus (aa1-30) of HBX is a well-conserved negative regulatory region (Ritter *et al.*, 1991; Arii *et al.*, 1992; Runkel *et al.*, 1993). Neither the amino- nor the carboxyl-termini (aa143-154) of HBX are required for transactivation (Arii *et al.*, 1992). There is no consensus as to which regions are required for transactivation. Arii *et al.* (1992) found the aa regions 46-52, 61-69 and 132-139 of HBX to be essential for transactivation. Similarly, Takada *et al.* (1994) demonstrated 3 sites that interact with cellular proteins and are essential for transactivation. These were aa regions 65-72, 105-115 and 131-142. However, Runkel *et al.* (1993) found only 2 regions essential for transactivation, namely, aa regions 61-69 and 110-139, (figure 5(c)).

HBX has been shown to be a multifunctional protein exhibiting a number of activities affecting transcription, cell growth and apoptotic cell death. HBX does not directly bind DNA, however it is considered to be a promiscuous transactivator of several cellular and viral promoters and enhancers (reviewed by Andrisani *et al.*, 1999). The transactivation mechanism postulated is that HBX enhances DNA binding potentials and transcription efficacies by interacting with various transcription factors. Additionally, from studies in transgenic mice, HBX induces hepatocellular carcinomas. This has lead to the conclusion that HBX may be involved in hepatocarcinogenesis (Arii *et al.*, 1992; Kim *et al.*, 1993; Kidd-Ljunggren *et al.*, 1995; Uchida *et al.*, 1997).

The X ORF overlaps with essential regions of the HBV genome: the direct repeat (DR)1 and DR2 (sequences required for genome replication), enhancer II, a core upstream regulatory sequence, a negative regulatory element and a major portion of the basic

core promoter (Ganem *et al.*, 1987;Carman *et al.*, 1992; Okamoto *et al.*, 1994; Thomas *et al.*, 1994; Kidd-Ljunggren *et al.*, 1995).

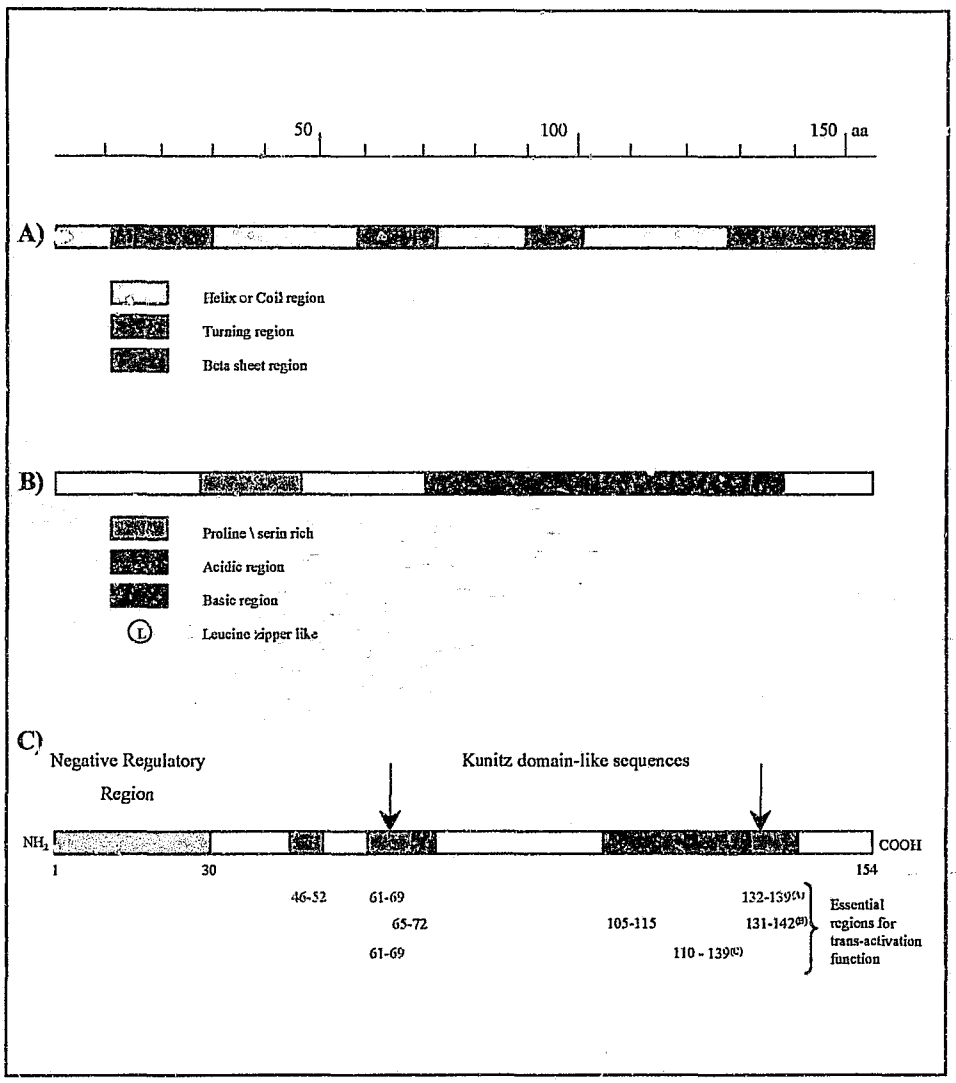


Figure 5 Schematic representation of the predicted secondary structure and characteristic regions in the HBV X protein.

A) Linear representation of the secondary structure of HBX

B) Amino acid sequence analysis of HBX

C) Essential and non-essential coding regions as reported by Ritter *et al.*, 1991[(B)]; Arii *et al.*, 1992[(A)]; Runkel *et al.*, 1993[(C)].

1.1.4.2.4 Precore/core and core genes

The precore/core and the core ORFs encode for 2 proteins, HBeAg and HBcAg, respectively, that are translated from two in-frame initiation codons (AUG), (figure 6(a)), (Carman *et al.*, 1993; Thomas *et al*, 1994; Carman, 1995; Howard, 1995). The synthesis of HBcAg is initiated from the second AUG initiation codon of the core gene. It is the major polypeptide of the nucleocapsid necessary for the packaging of the pregenomic RNA and subsequent particle formation, (figure 6(b)), (Akahane *et al.*, 1990; Carman *et al.*, 1992, 1993; Thomas, *et al.* 1994; Howard, 1995). HBeAg synthesis is initiated from the first AUG initiation codon found in the precore/core region. It produces a 212 aa protein (p25c) that undergoes extensive post-translational modification. p25c is cleaved at the nineteenth aa of the precore/core fusion protein by a signal peptidase, generating a p22 hydrophilic intermediate protein product. p22 is then either released back into the cytoplasm or is translocated to a RNA ribosomal complex in the endoplasmic reticulum. Here the carboxyl terminus is cleaved by proteolysis, producing a soluble p17 protein, known as HBeAg (figure 1.6(c)). HBeAg may then be secreted into the blood or be expressed on the hepatocyte surface (Akahane *et al.*, 1990; Bonino *et al.*, 1991; Carman *et al.* 1992; Schoub, 1994; Howard, 1995; Scaglioni *et al.*, 1997a).

HBeAg is not required for viral replication, but is thought to function as a tolerogen because it shares epitopes with HBcAg. It reduces the immune pressure on HBcAg by diverting the immune effector cells away from the infected hepatocytes, allowing for prolonged viral survival (Chang *et al.*, 1987; Schlicht *et al.*, 1987; Carman *et al.*, 1992, 1993; Thomas *et al.*, 1994; Carman, 1995).

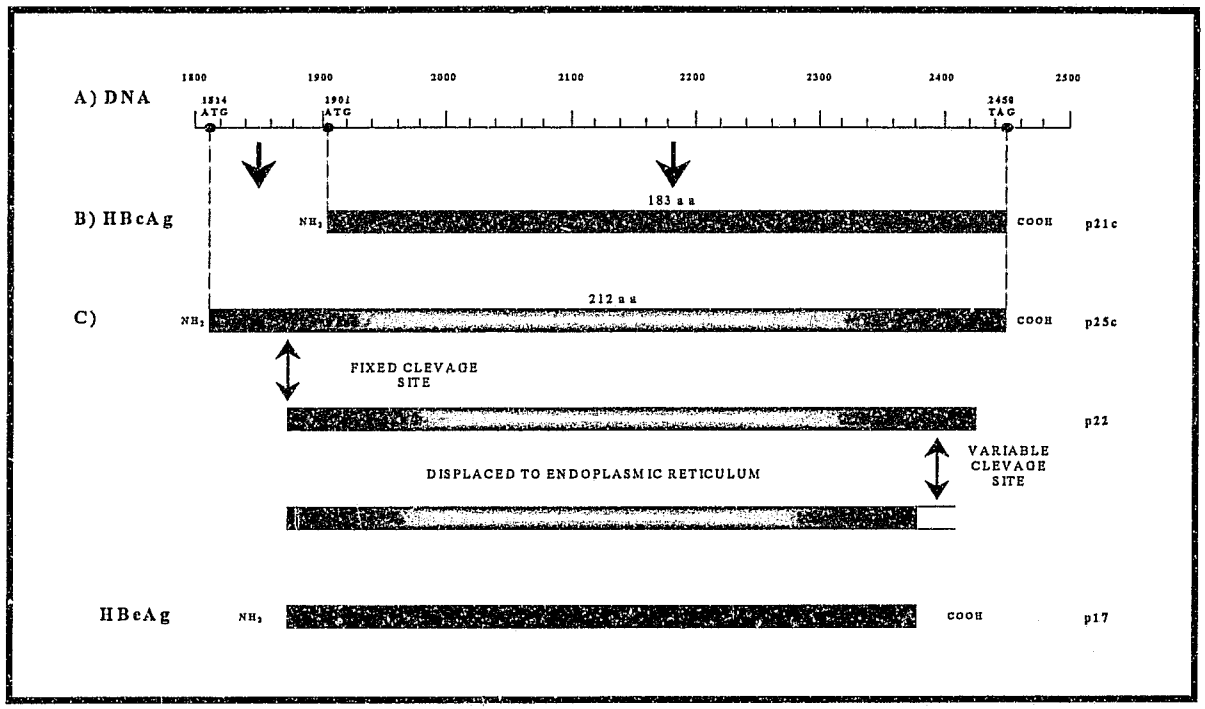


Figure 6 Translation products of the precore/core and core region of HBV

A) DNA strand of the precore/core and core region with its two start codons (AUG) and single stop codon (UAG).

B) Translation beginning at the second AUG produced HBcAg.

C) Translation beginning at the first AUG produces the p25c protein, which undergoes post-translational modification to produce HBeAg, (see text).

Because HBeAg is secreted into the serum it is used as a marker of infectivity and viral replication. Seroconversion to anti-HBe is indicative of decreased viral replication and recovery from disease (Bonino *et al.* 1991).

1.2 HBeAg-negativity and HBV infection

As discussed in section 1.1.4.2.4, seroconversion from a HBeAg-positive to a HBeAg-negative phenotype is traditionally associated with a decreased level of HBV replication (Bonino *et al.*, 1991). However, studies have revealed individuals who have seroconverted to anti-HBe (thus have a HBeAg-negative phenotype), but who still have active viral replication and severe disease.

Two hypotheses as to why this occurs have been proposed. The first theory suggests that the HBeAg precursor protein may inhibit HBV transcription and particle maturation. Thus, a lack of HBeAg would result in an increase of both viral transcription and particle formation allowing for active viral replication. The HBeAg precursor protein is speculated to inhibit HBV transcription by suppressing or down-regulating the activities of the viral enhancer elements. Particle maturation may be inhibited because there may be some kind of stoichiometric interaction between the core and precore proteins that prevents encapsidation and reverse transcription of the pregenomic RNA (Lambert *et al.*, 1993; Guidotti *et al.*, 1996). The second theory suggests that in early infection hepatocytes infected by HBeAg mutants may be inefficiently eliminated by the host's immune responses, thus allowing for widespread infection. However, at a later stage of infection, when the host's immune system is triggered by other viral components, massive hepatocyte destruction of the infected hepatocytes occurs, resulting in more

severe disease (Baumert *et al.*, 1996). Therefore, the loss of HBeAg is not always a true reflection of diminished viral replication but may result from the appearance of mutants.

HBeAg production may be affected at a transcriptional and/or a translational level. Initially, much attention was devoted at looking for mutations that interfere with HBeAg production at a translational level. Following sequencing of the precore/core region, a 1896 G to A stop codon mutation was identified which truncated the HBeAg, thus resulting in no HBeAg detection. Other mutations like non-functional start codons (conversion of AUG to AUA, AGG, UUG or UTT), nucleotide insertions or deletions leading to frameshift mutations and other stop codon mutations (e.g. 1817) have also been found to interfere with HBeAg's translation (reviewed in Miyakawa *et al.*, 1997). Subsequent studies revealed that these mutations did not occur in all HBeAg-negative cases and that the 1896 mutation had an uneven worldwide distribution. The 1896 stop codon mutation was frequently observed in HBV from Asian and Mediterranean patients, but was rarely found in Western Europe and North America (Kramvis *et al.*, 1997; Nagasaka *et al.*, 1998; Chan *et al.*, 1999). Because of the high HBeAg-negativity rate in southern African black adult carriers (who are at an increased risk of developing HCC), Kramvis *et al.* (1997) looked for the 1896 stop codon mutation in this population. They found that despite the high HBeAg-negative rate, it rarely occurred in this population (Kramvis *et al.* 1997; 1998a). From this and other studies, it was noted that the presence or absence of the 1896 stop codon mutation coincides with the differences in distribution of HBV genotypes (Kramvis *et al.*, 1997; Nagasaka *et al.*, 1998).

Nucleotide 1896 is located at the 5'end of the pregenomic RNA. For the pregenomic RNA to be encapsidated, this 5'end (nt1846 – nt1916) folds into a stem-loop structure, known as the encapsidation signal or epsilon. Epsilon's secondary structure consists of

a stable bipartite stem-loop structure containing a six-nucleotide bulge, a six-nucleotide loop and a single unpaired uracil residue (Junker-Niepmann *et al.*, 1990). The region encoding for epsilon has been found to be highly conserved between different genotypes of HBV, the only difference being at nucleotide 1858, in the ascending limb of the lower stem. This nucleotide can either be a thymine (T-1858) or a cytosine (C-1858). T-1858 forms a wobble pair with guanine G-1896 (U:G) in the descending limb, whereas C-1858 forms a stable Watson-Crick base pair with G-1896 (C-G) in the descending limb. Therefore, a G to A mutation at position 1896 would convert the original U:G wobble base pair in the lower stem of epsilon to a U-T Watson-Crick pair in genotypes with T-1858 but would destabilize the secondary structure of epsilon in genotypes with C-1858. It is known that T-1858 is found mostly in genotypes B, C, D and E, whereas C-1858 is found mainly in genotype A (reviewed by Kramvis *et al.*, 1998b; Lindh *et al.*, 1998;Nagasaka *et al.*, 1998; Chan *et al.*, 1999; Cho *et al.*, 1999; Blackberg *et al.*, 2000).

This single difference (C or T at 1858) thus accounts for the uneven distribution of the 1896 stop codon mutation in HBeAg-negative individuals restricting it to HBV genotypes with T-1858 (genotypes B, C, D and E). These genotypes are predominant in Asia and the Mediterranean basin and are rarely found in North America and Western Europe, where genotype A is the predominant genotype (Lindh *et al.*, 1998; Nagasaka *et al.*, 1998; Chan *et al.*, 1999; Cho *et al.*, 1999; Blackberg *et al.*, 2000). In southern Africa, the predominant genotype is genotype A. Therefore, the 1896 G to A mutation would not be expected to occur and does not frequently occur in this area (Kramvis *et al.*, 1997; 1998a).

1.3 Core promoter

The core promoter overlaps with the distal X and proximal precore/core ORFs and includes at least 232 base pairs (bp) (nucleotide (nt)1591 – nt1822), (numbering from *Eco*R1 site, Galibert *et al.*, 1979). Its functional elements include the upstream regulatory region (URR) (nt1613 – nt1742) the enhancer II region (nt1627 – nt1774) and the basic (basal) core promoter (BCP) (nt1742 – nt1849). The URR contains a negative regulatory element (NRE) (nt1613 – nt1636) and a core upstream regulatory sequence (CURS) (nt1636 – nt1742), (figure 7), (Yaginuma *et al.*, 1989; Yuh *et al.*, 1990, 1991; Guo *et al.*, 1993; Friedt *et al.*, 1999).

1.3.1 Negative regulatory element (NRE)

The NRE functions to down-regulate the activity of the core promoter in an orientation-independent but position-dependent manner (Gerlach *et al.*, 1992). It is capable of down-regulating the activity of the core promoter by repressing the stimulatory effect of the enhancer II in differentiated liver cells. Nucleotides 1616 to 1621 have been shown to be essential for this repression (Lo *et al.*, 1994; Park *et al.*, 1997).

1.3.2 Core Upstream Regulatory Sequence (CURS)

The CURS is a regulatory region that is able to activate the BCP (from a position and orientation dependent manner), in well-differentiated liver cells, but not in poorly differentiated or in non-liver cells. CURS has been subdivided into two domains, namely CURS-A (nt1636 – nt1703) and CURS-B (nt1704 – nt1743). CURS-A is capable of exerting a positive regulatory effect on the BCP on its own, although to a lesser degree

than the entire CURS (Wang *et al.*, 1990; Yuh *et al.*, 1991, 1992, 1993; Guo *et al.*, 1993).

1.3.3 Enhancer II

The HBV enhancer II is located downstream of enhancer I, within the X ORF, and has been mapped within nt1627 to nt1774 (Wang *et al.*, 1990; Yuh *et al.*, 1990, 1991; Zhou *et al.*, 1990). No consensus has however been reached about its minimal sequence and has been variously defined as nt1687 – nt1805 (López-Cabrera *et al.*, 1991), nt1627 - nt1732 (Wang *et al.*, 1990), nt1685 - nt1774 (Yee *et al.* 1989) and nt1646 - nt1741 (Lo *et al.*, 1994).

It has only been shown to be active in HCC cells and is thus speculated to contribute to the liver-tissue specificity of HBV (Wang *et al.*, 1990; Yen, 1993; Yuh *et al.*, 1993). It consists of 2 elements, II-A (nt1636 - nt1690) and II-B (nt1704 - nt1741). Co-operation of both these elements is necessary for the enhancer II to function (Yuh *et al.*, 1990; Wu *et al.*, 1992). Two interacting sequence motifs have been identified: a 23bp sequence (nt1646 - nt1668) within the II-A element, referred to as the α-box, and a 12bp sequence (nt1704 - nt1715) within the II-B element, referred to as the β-box. Both these sequences are indispensable and sufficient to retain the enhancer II's function, are able to regulate transcription from the BCP individually, and are able to bind multiple cellular factors (figure 7), (Yuh *et al.*, 1991; Yen, 1993; Li *et al.*, 1995). The enhancer II is capable of upregulating the surface and X promoters (from a downstream orientation- and position-independent manner), activate the BCP (from an upstream position- and orientation-dependent manner) and aids in conferring liver-tissue specificity for HBV (Yuh *et al.* 1990; Zhou *et al.*, 1990; Yen, 1993).

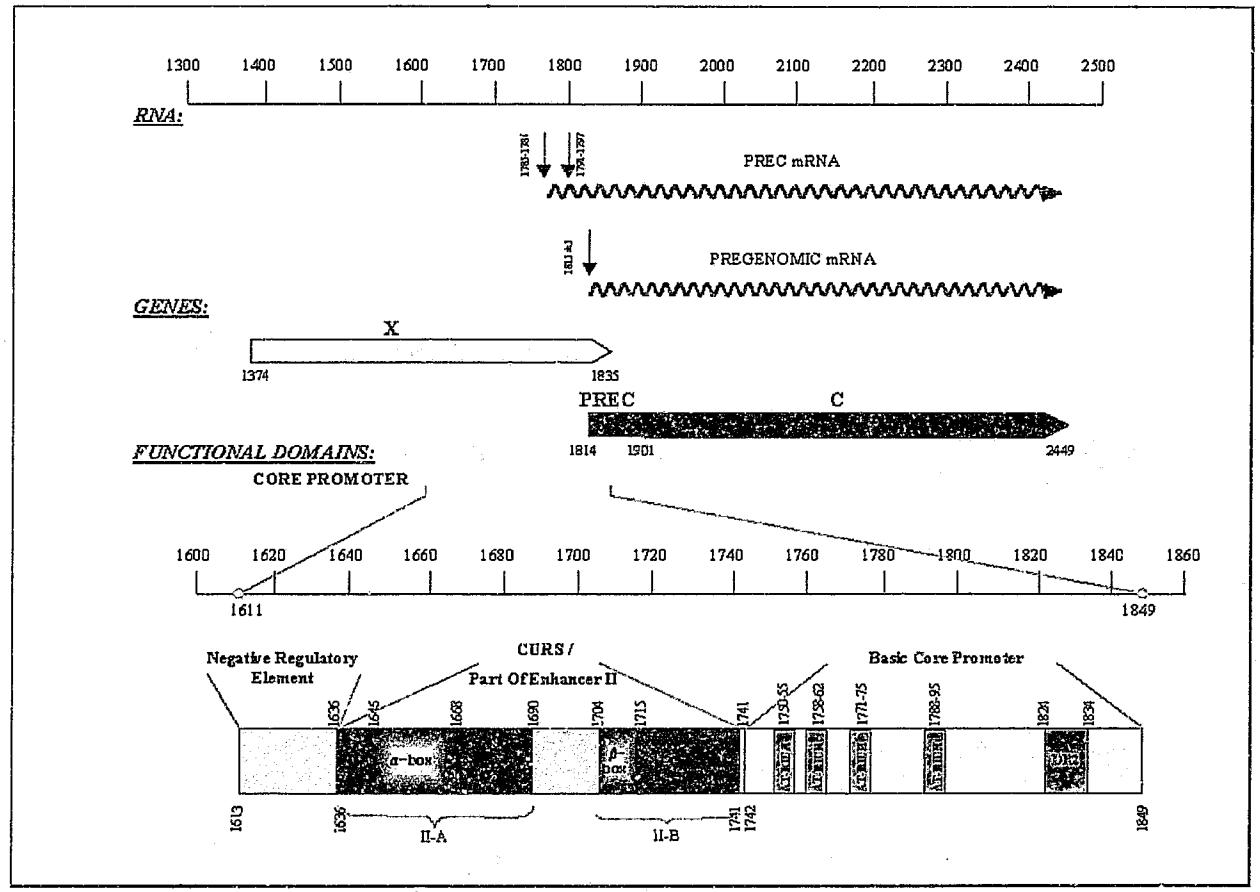


Figure 7 Schematic representation of the core promoter, including the negative regulatory element, enhancer II and basic core promoter.

1.3.4 Basic core promoter

A 108-bp region (nt1742 to nt1849) was initially determined to be sufficient for the correct initiation of both the precore and pregenomic mRNAs (Yaginuma *et al.*, 1989; Yuh *et al.*, 1992). However, analysis of deletion mutants by Chen *et al.* (1995a) and Yu *et al.* (1996) have more accurately defined this region.

1.3.4.1 Structure

Typically, RNA polymerase II promoters consist of a TATA-box motif [TATAAA] situated approximately 30bp upstream of the transcription initiation site and/or an initiator that overlaps the transcription start site. Both TATA and initiator elements are capable of directing initiation of transcription (Yu *et al.*, 1996).

The BCP, even though a RNA polymerase II promoter, lacks distinct TATA-box motifs. Four AT-rich regions, within this region, have been found and hypothesized to assume the role of TATA boxes (Chen *et al.*,1995a; Yu *et al.*, 1996). These AT-rich regions are AGATTA (nt1750 – nt1755), TTAAA (nt1758 – nt1762), TATTA (nt1771 – nt1775) and CATAAATT (nt1788 – nt1795), (figure 7), (Yaginuma *et al.*, 1987; Chen *et al.* 1995a; Honda *et al.* 1999).

Because, 1750-AGATTA-1755, 1758-TTAAA-1762 and 1771-TATTA-1775 are located 20-35bp from the transcription initiation site of the precore mRNA (nt1785 to nt1786 and nt1791 to nt1797), it has been speculated that they function as TATA motifs for this mRNA. Similarly, 1788-CATAAATT-1795 is located approximately 30bp from the transcription initiation site of the pregenomic mRNA (nt1815 ± 5) and thus it has been considered to function as the TATA motif for this mRNA (Will *et al.*, 1987; Yaginuma *et*

al., 1987; Honigwachs *et al.*, 1989; Hiraga *et al.*, 1994; Chen *et al.*, 1995a; Günther *et al.*, 1996). Additional support that these AT-rich regions are TATA-like boxes comes from the fact that recombinant TATA-binding protein (TBP) is able to bind to all 4 regions, as determined by both footprint and competitive gel mobility-shift assay (Chen, *et al.*, 1995a; Yu *et al.*, 1996).

Using mutant deletion analysis, Chen *et al.* (1995a) have shown that of the 108bps, a 15 nt sequence from nt1788 to nt1802 is sufficient to precisely direct the initiation of both precore and pregenomic RNAs. This region includes the initiation site for precore but not pregenomic RNA. From their results they concluded that this region has the dual function of being an initiator for the transcription of the precore RNA and a TATA element for the pregenomic RNA (Chen *et al.*, 1995a).

Yu *et al.* (1996) confirmed and expanded on these results when they reported that the synthesis of the precore and pregenomic mRNAs is under the control of two distinct promoters spanning this region. They found that each of the promoters responsible for the synthesis of precore and pregenomic mRNAs, each has its own TATA and initiator sequences. Even though these promoters overlap slightly, they have been reported to be genetically separable. The precore promoter spans the sequence between nt1758 and nt1791. They speculated 1758-TTAAA-1762 to be the TATA-like sequence and 1788-CATA-1791 the initiator sequence (since its overlaps initiation sites of the precore and resembles the optimal initiator sequence 5'-CA(T/G)T-3'). The pregenomic promoter spans the sequence between nt1788 and nt1821. They speculated that 1788-CATAAAT-1794 acts as the TATA-box sequence and 1817-CAACT-1821 as the initiator sequence (which overlap the transcription initiation sites of the pregenome) (Yu *et al.*, 1996).

1.3.4.2 Functions

The BCP is central to the life cycle HBV because it regulates the transcription of two types of 3.5kb transcripts, the precore and pregenomic mRNAs. Even though these transcripts only differ by about 30 nucleotides at their 5'ends, they are functionally distinct producing a RNA template for genome replication and viral proteins (Schaller *et al.*, 1991; Yuh *et al.*, 1992; Okamoto *et al.*, 1994; Chen *et al.*, 1995a; Sato *et al.*, 1995; Baumert *et al.*, 1996; Scallion *et al.*, 1997a, b).

The precore mRNA is longer than the pregenomic mRNA, is initiated 20 to 30 nucleotides downstream of the precore ORF, and is translated into the precore/core protein that is processed to produce the HBeAg, (see section 1.1.4.2.4). The 5' end of the pregenomic mRNA is located 5 nts downstream from the precore initiation codon and is indispensable for viral genome replication. Most of this mRNA is translated into the core and polymerase proteins, while a small fraction is encapsidated into core particles and reverse transcribed into the viral DNA genome (Schaller *et al.*, 1991; Yuh *et al.*, 1992; Okamoto *et al.*, 1994; Chen *et al.*, 1995a; Sato *et al.*, 1995; Baumert *et al.*, 1996; Scaglioni *et al.*, 1997a, b). As discussed in section 1.3.3.1, their transcription is regulated separately.

Baumert *et al.* (1996, 1998) identified a novel function of the core promoter in the regulation of core protein expression. They suggested that it regulates core protein synthesis at a transcriptional or posttranscriptional level without affecting RT-polymerase expression. The molecular mechanism whereby this occurs is however not completely understood.

1.3.4.3 Mutations reported in the BCP

The BCP has been found to be fairly heterogeneous, having both highly conserved and hypervariable regions. The regions between nt1776 - nt1798 and nt1812 - nt1837 were found to be highly conserved, whereas the regions between nt1751 - nt1775 and nt1799 - nt1811 are hypervariable (Kidd-Ljunggren *et al.*, 1997).

A number of changes resulting in silent, missense and nonsense mutations have been reported in patients with varying disease status from all over the world. What has become apparent is that regardless of the type of clinical profile, the changes in the BCP are similar, if not identical. What differs, is the frequency with which these changes occur.

Tables 1 and 2 give a summary of the most commonly observed BCP mutations in natural pathogenesis, as reported by different researchers.

Table 1 Most common point mutations reported in the BCP.

MUTATION	EFFECT ON X ORF	HBV CLINICAL MANIFECTATION	PATIENT ETHNICITY	REFERENCE
1753 T→C	Missense mutation at aa 127	HCC	Japanese	Takahashi *et al.*, 1998
		Chronic HBV infection	Japanese	Nagasaka *et al.*, 1998
		Chronic liver disease	Japanese	Takahashi *et al.*, 1999
		Chronic liver disease	Japanese	Honda *et al.*, 1999
1762 + 1764 A→T + G→A	Missense mutations at aa 130 and 131	Chronic HBV infection – ASC	Japanese	Okamoto *et al.*, 1994
		ASC	Japanese	Moriyama *et al.*, 1994, 1996
		ASC	Swedish	Lindh *et al.*, 1998; 1999
		Chronic carriers	Swedish	Kidd-Ljunggren *et al.*, 1995, 1997
		Chronic liver disease	Japanese	Takahashi *et al.*, 1995, 1999
		Chronic liver disease	German	Günther *et al.*, 1996
		Chronic HBV infection	Japanese	Nagasaka *et al.*, 1998
		Chronic liver disease	Japanese	Honda *et al.* 1999
		Chronic HBV infection	Chinese	Chan *et al.*, 1999
		Childhood chronic HBV infection	Japanese	Ando *et al.*, 1999
		Chronic HBV infection	Swiss	Mayerat er at., 1999
		Chronic HBV infection	Japanese	Shindo *et al.*, 1999
		Chronic HBV infection	Chinese	Hou *et al.*, 1999
		Chronic HBV infection	Korean	Kim *et al.*, 1999
		Seroconverting HbeAg HBV infection	Swedish	Bläckberg *et al.*, 2000
		Fulminant hepatitis	Japanese	Ogata *et al.*, 1993
		Fulminant hepatitis	United States	Hasegawa *et al.*, 1994
		Fulminant hepatitis	Japanese	Horikita *et al.*, 1994
		Fulminant hepatitis	Japanese	Sato *et al.*, 1995
		Fulminant hepatitis	Japanese	Kaneko *et al.*, 1995
		Fulminant hepatitis	Italian, German, Turkish	Sterneck *et al.*, 1996
		Fulminant hepatitis after heart transplantation	Donor = Asian origin	Pult *et al.*, 1997
		Fulminant hepatitis	Japanese	Inoue *et al.* 1998
		Fulminant hepatitis	German	Friedt *et al.*, 1999
		HCC	German	Loncarevic *et al.*, 1990
		HCC	N/D	Blum *et al.*, 1991
		HCC	Korean	Cho *et al.*, 1999
		HCC	Chinese	Hsia *et al.*, 1996
		HCC	Japanese	Takahashi *et al.*, 1998
		HCC	Chinese	Fang *et al.*, 1998
		Chronic HBV infection and ASC	Japanese	Nishizono *et al.*, 1995
		Anti-HBe positive chronic hepatitis	Italian, Turkish, Afghan	Tong *et al.*, 1990
		ASC (HBeAg-negative) and chronic HBV infection	Japanese	Kurosaki *et al.*, 1996
		Fulminant hepatitis and self-limited hepatitis	United States	Laskus *et al.*, 1995
		Recurrent HBV infection post-transplantation	N/D	McMillan *et al.*, 1996
1824 T→C		"silent" HBV	Eastern Japanese	Uchida *et al.*, 1994a
1766 + 1768 C→T + T→A	Missense mutations at aa 132 and 133	Fulminant hepatitis	N/D	Baumert *et al.*, 1996
		Chronic active HBV	German	Gerner *et al.*, 1999

Table 2 Most common deletions and insertions reported in the BCP.

MUTATION	EFFECT ON X ORF	HBV CLINICAL MANIFECTATION	PATIENT ETHNICITY	REFERENCE
Deletion 1755-1762 (8bp)	Truncated X protein	HCC	Chinese	Hsia *et al.*, 1997
Deletion 1758-1765 (8bp)	Truncated X protein	Immunologically negative for HBV markers	German	Preisler-Adams *et al.*, 1993
Deletion 1762-1769 (8bp)	Truncated X protein	HCC	Chinese	Hsia *et al.*, 1997
Deletion 1763-1770 (8bp)	Truncated X protein	Chronic HBV infection – ASC	German	Repp *et al.* 1992
		Chronic HBV infection	United States	Laskus *et al.*, 1994a
		Recurrent HBV infection after orthotopic liver transplantation	United States	Laskus *et al.*, 1994b
		ASC	Japanese	Horikita *et al.*, 1994
		Chronic active HBV infection	Swiss	Mayerat *et al.*, 1999
Deletion 1768-1775 (8bp)	Truncated X protein	"silent" HBV; serologically negative acute/chronic HBV	Eastern Japanese	Uchida *et al.*, 1994a, 1995
		ASC vs. severe chronic active hepatitis; serologically "silent" non-B, non-C chronic patients	Western Japanese	Fukuda *et al.*, 1995, 1996
		ASC	Japanese	Gotoh *et al.*,1995
Deletion 1756-1773 (18bp)	Truncated X protein	Chronic HBV infection	United States	Laskus *et al.*, 1994a
Deletion 1748-1768 (21bp)	Truncated X protein	Chronic HBV infection	United States	Laskus *et al.*, 1994a
Deletion 1750-1770 (21bp)	Truncated X protein	Chronic persistent hepatitis	Japanese	Horikita *et al.*, 1994
Deletion 1755-1774 (21bp)	Truncated X protein	Chronic HBV infection	United States	Laskus *et al.*, 1994a
Variable length deletions between 1742-1780	Truncated X protein	Chronic liver disease	German	Gunther *et al.*, 1996
Variable length deletions between 1753-1777	Truncated X protein	Chronic HBV infection – ASC	Japanese	Okamoto *et al.*, 1994
Variable length deletions between 1756-1775	Truncated X protein	ASC	Turkish	Barlet *et al.*, 1994
Insertion between 1820 and 1821 (5bp)	Frameshift mutation resulting in fusion of X protein to the precore/core region	Immunologically negative for HBV markers	German	Preisler-Adams *et al.*, 1993
Insertion between 1775 and 1776 (11bp)	Truncated X protein	Chronic HBV infection	United States	Laskus *et al.*, 1994a
Insertion between 1775 and 1776 (11bp)	Truncated X protein	Immunosuppressed heart transplant patient who developed fulminant hepatitis	N/A	Pult *et al.*, 1997
Insertion between 1777 and 1778 (12bp)	Truncated X protein	Recurrent HBV infection after orthotopic liver transplantation	United States	Laskus *et al.*, 1994b
Insertion between1767-1769 (3bp)	Truncated X protein	Chronic active hepatitis	Swiss	Mayerat *et al.*, 1999
Variable length insertions between 1721-1790	Truncated X protein	Chronic liver disease	German	Gunther *et al.*, 1996

1.3.4.4 *In vitro* consequences of the common HBV mutations

A number of *in vitro* studies have been undertaken using HBV variants with the most common naturally occurring mutations (as summarized in tables 1 and 2). The reported consequences of these mutations are not always in agreement. The inconsistencies may result from the different plasmid constructs and cell lines used.

Table 3 summarizes the different phenotypic effects observed by various researchers, when specific mutations were introduced into the BCP of HBV.

1.3.4.5 Transcription factors binding to the BCP

A dense clustering of protein binding sites has been reported to occur along the BCP region. Binding of several *cis*-acting elements, both liver-specific and ubiquitous, is essential for liver-specific expression of HBV transcription (Johnson *et al.*, 1995). Table 4 summarizes the different transcription factors reported to bind to the core promoter.

Nucleotide changes in the BCP DNA may result in inefficient nuclear protein binding, enhanced nuclear protein binding or even create new nuclear protein binding sites that may alter HBV transcription and protein synthesis (Li *et al.*, 1995; Günther *et al.*, 1996; Pult *et al.*, 1997; Raney *et al.*, 1997; Li *et al.*,1999).

Table 3 *In vitro* consequences of the common HBV mutations.

Mutation(s) functionally tested	Phenotypic effect(s) on HBV	Reference
1762 + 1764	enhanced replication	Hasagawa *et al.*, 1994
1762 + 1764	no significant difference in promoter activity when comparing mutant and wildtype HBV	Nishizona *et al.*, 1995
1762 +1764	enhanced replication did not affect level of pregenomic mRNA reduced transcription of precore mRNA and HBeAg no change in HBcAg and HBsAg protein levels	Buckwold *et al.*, 1996
1762 +1764	enhanced replication increased HBcAg production reduced levels of precore mRNA, resulting in reduced levels of HBeAg	Moriyama *et al.*, 1996
1762 + 1764	enhanced viral replication	Pult *et al.*, 1997
1766 +1768	enhanced encapsidation and replication minor increase in transcription of pregenomic mRNA increased HBcAg expression	Baumert *et al.*, 1998
1762 +1764 +1766 +1768	viral replication moderately enhanced did not affect synthesis of pregenomic mRNA decreased precore mRNA and HBeAg no change to HBsAg and HBcAg levels	Scaglioni *et al.*, 1997b
1653 (C to T) + 1762 (A to T) + 1764 (G to A)	no enhanced viral replication did not affect level of pregenomic mRNA, decreased precore mRNA and HBeAg no change in HBsAg and polymerase levels	Günther *et al.*, 1998
8 bp deletion (1763 – 1770)	replication competent weak precore promoter activity increased core transcripts produced reduced amounts of HBcAg, HBeAg and HBsAg	Moriyama *et al.*, 1997
20bp deletion (nt1753 – nt1772)	replication competent completely abolishes precore promoter activity increased core transcripts produced reduced amounts of HBcAg, HBeAg and HBsAg	Moriyama *et al.*, 1997

Table 4 Transcription factors binding to core promoter.

Transcription factor(s)	Transcription factor binding site(s)	Reference
C/EBP (CCAAT/enhancer binding protein)	nt1642 – nt1664 nt1673 – nt1688 nt1745 – nt1764 nt1804 – nt1825 nt1835 – nt1848	López-Cabrera *et al.*, 1990
C/EBP-related protein(s) (but not C/EBP)	α-box and β-box of enhancer II	Yuh *et al.*, 1993
COUP-TF1, HNF4, TR2, PPAR-α-RXR-α, PPAR-γ-RXRα	nt1749 – nt1773 (DR1 hormone receptor element)	Buckwold *et al.*, 1997; Raney *et al.*, 1997; Yu *et al.* 1997
HNF3	nt1678 – nt1789 nt1713 - nt1724	Johnson *et al.*, 1995
HNF3	nt1716 – nt1724 (enhancer II)	Li *et al.*, 1995
HNF3	nt1713 – nt1724	Günther *et al.*, 1996
HNF4	nt1660 – nt1672 nt1755 – nt1767	Guo *et al.*, 1993; Raney *et al.*, 1997
RLNE (rat liver nuclear extract)	nt1619 – nt1630 nt1635 – nt1701 nt1706 – nt1741 nt1747 – nt1775 nt1799 – nt1825 nt1829 – nt1848	López-Cabrera *et al.*, 1990
Liver-enriched protein factor (LEF)	nt1759 – nt1769	Buckwold *et al.*, 1996
SP1	nt1621 – nt1630 nt1731 – nt1740 nt1742 – nt1752	Zhang *et al.*, 1993
SP1	nt1731 – nt1740 nt1743 – nt1754	Chen *et al.*, 1995b
SP1	nt1731 – nt1740 nt1743 - nt1752	Günther *et al.*, 1996
TBP, liver-specific factor	nt1758 – nt1776	Günther *et al.*, 1996
TBP	nt1788 – nt1795	Günther *et al.*, 1996
Sites for Tope I breakage	nt1759 – nt1766	Laskus *et al.*, 1997
Unidentified transcription factors	nt1711 – nt1727 nt1758 – nt1776	Zhang *et al.*, 1994

1.4 Hepatocellular Carcinoma and HBV infection

HCC is one of the most common tumours in many parts of the Far East and sub-Saharan Africa, with more than 310000 people developing the tumour each year (Bosch *et al.*, 2000). High incidences of the tumour (>15/100000 of the population per annum) have been reported in Africa (south of the Sahara desert), Taiwan, Hong Kong, Japan, Korea, Malaysia, Philippines, Singapore and Burma. Low incidences of the tumour (<5/100000 of the population per annum) have been reported in North America, Iceland, Greenland, most of Canada, Great Britain, Australia, New Zealand, Scandinavia, Israel, India and Sri Lanka. The remaining countries either have an intermediate incidence, or have an undocumented incidence (Kew, 1994; 1996).

The geographic distribution of HCC, at both local and global levels, is strikingly similar to that of the HBV carrier state. This, together with the fact that HBV DNA has been found in as much as 80% of HCC patients in high incidence regions, is evidence that it may directly or indirectly contribute to the aetiology of this cancer. Other major environmental factors that have been implicated in the causation of HCC include chronic infection with the hepatitis C virus, cirrhosis and exposure to the mycotoxin, aflatoxin B1 (Kew, 1994; 1996).

Black southern Africans are exposed to and generally get infected with HBV, in the first months of life. Because only 1 to14% of pregnant black carriers have replicative infection (HBeAg-positive), infection of their infants is mostly by the horizontal route. This results in a persistent, asymptomatic carrier state in 80-90% of these children. Male carriers have a lifetime relative risk of more than 100 of developing HCC, and it

has been estimated that approximately 40% of HBV carriers will die as a result of this tumour or cirrhosis or both (Kew, 1994; 1996).

In southern African blacks, HCC is frequently encountered in young adults in their second or third decade of life. The disease often has an acute onset, and runs a fulminant course. The tumour responds poorly to cancer chemotherapeutic agents and is seldom resectable.

1.5 Rationale and aims of the study

As already mentioned, HBV is endemic in southern African blacks. A unique feature of this population is that only 5% of HBV carriers are HBeAg-positive in adulthood as opposed to a higher rate of 40% found in other hyperendemic areas of the world. Previous studies carried out by Kramvis *et al.* (1997, 1998a) showed that in the Black population the high HBeAg-negative phenotype cannot be attributed to mutations affecting its translation. Therefore, the purpose of this study was to examine the nucleotide sequence of the BCP and the adjacent enhancer II region. Mutations within this region could affect HBeAg synthesis at the transcriptional level and cause the HBeAg-negative phenotype in black African carriers of HBV that may contribute to hepatocarcinogenesis in this population. Also, because these regions fall within the distal portion of the X gene and because the X gene is thought to play an important role in HBV induced HCC, the amino acid sequence of HBX was analyzed for mutations.

Chapter 2: MATERIALS AND METHODS

2.1 Subjects

Serum and liver tissue samples of HBsAg-positive southern African blacks were used.

2.1.1 Serum samples

Serum samples were obtained from 111 HBsAg-positive southern African blacks: 52 were asymptomatic carriers (ASC) with normal serum alanine transaminase levels, and 59 were patients with HCC.

The serum of 15 of the ASCs was HBeAg-positive and that of 37 was HBeAg-negative/anti-hepatitis B e (HBe)-positive. Serum of 25 of the HCC patients was HBeAg-positive and that of 34 patients was anti-HBe positive. The ratio of HBeAg- positive to anti-HBe positive samples, for both the ASC and HCC patients, was roughly one-third to two-thirds.

All markers for HBV were detected using commercially available kits (Abbott Laboratories, Chicago, IL). Serum samples were stored at -70°C until analyzed.

2.1.2 Tissue samples

All tumorous and nontumorous liver tissues were obtained at necropsy or laparotomy, 'snap frozen' in liquid nitrogen and then stored at -70°C until analyzed.

Tumorous and corresponding non-tumorous liver tissues were obtained from 10 patients. Tumorous tissue alone was obtained from a further 19 patients.

Seven of the tumorous/non-tumorous tissue pairs were obtained from patients whose serum was HBsAg-positive/HBeAg-negative and 3 from those who were anti-hepatitis B surface (HBs)/anti-hepatitis B core (HBc) positive. Serum in the majority of the patients (16 of 19) from whom only tumorous tissue was obtained was HBsAg-positive/HBeAg-negative, in 2 patients anti-HBs/anti-HBc-positive, and in one patient anti-HBc- positive alone.

2.2 DNA extractions

The study was carried out on DNA extracted from serum and liver tissues samples. DNA was extracted from serum using the QIAamp blood kit and from the liver tissue using the salting-out protocol.

2.2.1 DNA extraction from serum

Total DNA (genomic and viral) was extracted from serum using the QIAamp blood kit (Quigen Inc., Hilden, Germany) and the protocol followed was that provided by the manufacturer.

Two hundred microliters of serum was pipetted into a sterile 1.5ml-microfuge tube. Twenty five microliters of Qiagen protease and 200µl buffer AL were added to the serum, mixed immediately by vortexing for 15 seconds, and incubated at 70°C for 10 minutes. This caused the lysis of viruses in the serum, thus allowing their DNA to be

released. The lysate was then incubated for 15 minutes at 95°C to inactivate any potential infectious agents that may have been present in the serum. Two hundred and ten microliters of 100% ethanol were added to the lysate and mixed by vortexing.
A QIAamp spin column was placed in a 2ml-collection tube, the mixture applied to it and centrifuged at 8000rpm for 1 minute. The brief centrifugation allowed for the adsorption of the DNA onto the QIAamp silica membrane. Proteins and other contaminants in the lysate were not adsorbed onto the membrane because of the salt and pH conditions used. To avoid cross-contamination between samples, care was taken not to moisten the rim of the spin column and column caps were closed carefully.

The QIAamp spin column was then washed twice with 500µl of buffer AW. Each time a clean 2ml-collection tube was used and the collection tube containing the filtrate discarded. These washes allowed for the removal of any residual contaminants without any effect on DNA binding. The QIAamp column was then placed in a sterile 1.5ml microcentrifuge tube, 50µl of preheated water (70°C) added to the QIAamp spin column, incubated at room temperature for 1 minute, and the DNA eluted from the column by a 1 minute, 8000rpm centrifugation. The eluted DNA was stored at 4°C.

2.2.2 DNA extraction from liver tissue

DNA from tumorous and non-tumorous tissue was extracted using a modified salting out procedure described by Miller *et al.* (1988). This protocol allows for efficient deproteinization by salting out cellular proteins. This is achieved by dehydration and precipitation with a saturated NaCl solution (Miller *et al.*, 1988).
One hundred milligrams of tissue was cut up and suspended in 5ml of buffer A, 200mg/ml proteinase K, and 10% sodium dodecyl sulfate (SDS). The tissue pulp was

digested for 2-3 hours at 37°C with continuous mixing. Two milliliters of saturated NaCl (approximately 6mol/l) was added to the sample and shaken for 15 minutes at room temperature. The sample was centrifuged for 15 minutes at 3500rpm and the supernatant transferred to a clean polypropylene tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, and the sample was mixed gently for 15 minutes at room temperature. The sample was then centrifuged at 3500rpm for 10 minutes at 8°C. The supernatant was transferred to a clean polypropylene tube, an equal volume of chloroform added and the solution was mixed gently for 5 minutes. The sample was centrifuged at 3500rpm for 10 minutes at 8°C, and the supernatant transferred to a clean polypropylene tube. Isoproponol was added gradually until the DNA precipitated out of solution. The precipitated DNA strands were removed with a pipette, washed for 2 minutes in 70% ice-cold ethanol, and resuspended in 200µl TE buffer. The resuspended DNA was stored at 4°C.

2.3 Polymerase chain reaction (PCR)

The primers pairs used to amplify the core promoter/enhancer II region in this study were designed with the aid of the 'oligo' (Wojciech Rychlik) and 'primer detective' (CLONTECH Laboratories, Inc., USA) programs. The HBV consensus sequence was used as a template. The core promoter/enhancer II region was amplified using a nested PCR, using primers mapping nts 1661 to 1921, (table 5). A nested PCR was necessary because of the low viral titers in the samples.

PCR was performed in 25µl and 50µl final reaction volumes for first and second rounds, respectively. The reaction mixture consisted of 0.01U/µl DynaZyme DNA polymerase (Version 2.0; Finnzymes OY, Espoo, Finland), 200µmol/l of each of the dNTPs, 1µmol/l

of each of the primers, and 50mmol/l KCl, 10mmol/l Tris HCl (pH 8.8), 1.5mmol/l $MgCl_2$, and 0.1% triton-X 100. Both rounds of PCR were performed in a programmable thermal cycler (Perkin Elmer, Norwalk, CT).

The cycle profile for the first round was 94°C for 30 seconds for denaturation, 60°C for annealing, and 72°C for 50 seconds for elongation, for 40 cycles, followed by a final extension step of 10 minutes at 72°C. For the second round of the nested PCR, the first round amplicon was diluted (1:100) and 5µl of this was reamplified using the inner primers. The cycling conditions were as for the first round of PCR except that annealing was performed at 45°C for 30 seconds.

Table 5 Oligonucleotides used in this study.

Primer	Sequence	Position (nt)	Size (bp)
Outer primers			
1622 (+)	5'GAACGCCCATCAGATCCTGC3'	1622 – 1641	344
1966 (-)	5'GTCAGAAGGCAAAAACGAGAG3'	1966 – 1946	
Inner primers			
1661 (+)	5'GACTCTTGGACTCTCAGC3'	1661 – 1678	260
1921 (-)	5'TTTATACGGGTCAATGTC3'	1921 – 1904	
Sequencing primer			
1897R	5'CCAAAGCCACCCAAGGCACAGC TTGGAGGCTT3'		

Best quality water, instead of DNA, positive and negative controls were included in both rounds of PCR. Sera positive for HBsAg, HBeAg, and HBV (by slot-blot hybridization) were used as positive controls. Sera from healthy blacks lacking all HBV markers were used as negative controls. To avoid cross contamination and false-positive results the precautions and procedures suggested by Kwok and Higuchi (1989) were strictly adhered to. The DNA was extracted in a physically separate venue from where the PCR was performed. Separate sets of supplies and pipetting devices were dedicated for sample preparation and setting up of PCR reactions. Furthermore, PCR reactions were set up in a different venue from where the sample DNA was added to the reaction. The sample DNA was always added last to minimize the number of opportunities for the inadvertent transfer of DNA between samples.

2.4 Detection of amplified products

A 5µl aliquot of the amplified second round PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide. A 260bp band was visualized under ultraviolet light.

A 5, 10, 20 and 40ng/5µl DNA lambda ladder was run, on each agarose gel, to quantify each positive PCR sample, before sequencing.

Electrophoresis was performed at a venue physically isolated from the DNA extraction bench, PCR bench and DNA-addition bench to avoid contamination.

2.5 Nucleotide sequencing of PCR products

The Sanger-Coulson chain termination DNA sequencing method was used to sequence the core promoter/enhancer II region. The Sequenase PCR product sequencing kit (United States Biochemical Corp., Cleveland, OH) was used to directly sequence all samples.

2.5.1 Enzymatic pre-treatment of PCR product

Enzymatic pre-treatrnent of PCR products was necessary to remove excess primers, dNTPs and DNA polymerase which are in excess in the PCR reaction. If these components are not removed prior to sequencing, they may interfere with normal sequencing methods that also make use of primers and nucleotides. Shrimp alkaline phosphatase and Exonuclease I were the two hydrolytic enzymes used to achieve this. Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The shrimp alkaline phosphatase removes the remaining dNTPs from the PCR mixture that would interfere with the labeling step of the sequencing process.

An aliquot of the PCR amplification mixture (0.2 - 0.5pmol) was put into a 0.5ml-microcentrifuge tube. One microlitre of the Exonuclease I and 1µl of the shrimp alkaline phosphatase was used per 5µl of PCR product. The reaction was mixed, briefly centrifuged and incubated at 37°C for 15 minutes. The enzymes were inactivated by incubating the reaction at 80°C for 15 minutes. The DNA was then ready for direct sequencing.

2.5.2 Annealing template and primer

To a 0.5ml-microcentrifuge tube, 0.5pmol (1-9µl) of treated PCR product DNA and 10mM of either primer 1661 or 1921 was added. The volume was adjusted to 10µl with best quality water. Each sample was sequenced in the forward (primer 1661) and in the reverse (primer 1921) directions. In a few samples sequencing in the reverse direction with primer 1661 proved difficult, and in those cases primer 1897R was used instead (table 5).

The reaction was denatured at 99°C for 2 minutes and cooled as quickly as possible by placing the vial directly on an ice/water bath for 5 minutes. Rapid cooling promoted primer annealing over template re-annealing.

The tube was then centrifuged briefly and placed on ice until ready to begin labelling the reaction.

2.5.3 Labelling and termination reactions

While the template and the primer were annealing on ice, a microtiter plate was labelled and its wells coated with 2.5µL of each termination mixture (dideoxyl(dd)G, ddA, ddT, ddC). To the ice-cold annealed DNA mixture (10µL) the following reagents were added: 2µl of the reaction buffer (200mM Tris-HCl, pH7.5; 100mM $MgCl_2$; 250mM NaCl), 1µl dithiothreitol (0.1M), 2µl diluted labeling mix (1:10) (7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP), 0.5µl [^{35}S] dATP (5µCi) and 2µl Sequenase Version 2.0 T7 DNA polymerase.

These were mixed thoroughly and incubated at room temperature for 2 minutes. Simultaneously, the microtitre plate, coated with the dideoxynucleotides, was pre-warmed for 1 minute at 42°C.

At the end of the 2 minute labeling incubation period, 3.5µl of the labeled reaction was placed into each of the microtitre plate wells (incubating at 42°C) containing ddGTP, ddATP, ddTTP and ddCTP, respectively. The reaction was allowed to incubate at 42°C for 5 minutes.

The reactions were terminated by adding 4µl of the stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF).

The microtitre plates were stored at -20°C until ready for loading on the sequencing gel.

2.5.4 Denaturing gel electrophoresis

The samples were electrophoresed on 8% glycerol tolerant sequencing gels. Prior to loading, sequenced samples were denatured for 2 minutes at 83°C. Three to 3.5µl of each sample was loaded and electrophoresed for 2-3 hours at 60W.
The gels were 'fixed' for 20 minutes with fixing solution, dried and autoradiographed.

2.6 Statistics

Chi (χ^2), Fisher's exact and Student t tests were used to analyse the data. In every case only p values of less than 0.05 were considered to represent statistically significant differences.

Chapter 3: RESULTS

3.1 Detection of PCR products

Specific bands of the X/precore region of expected size (260 bps), (figure 8) were detected in agarose gels following amplification by nested PCR in all HBeAg-positive sera from 15 ASC and 25 HCC patients. On the other hand, a lower proportion of HBeAg-negative sera were HBV DNA positive by nested PCR: 32/37 (86%) ASC and 25/34 (73,5%) HCC serum samples. All of the tumorous tissues and non-tumorous tissues were PCR positive.

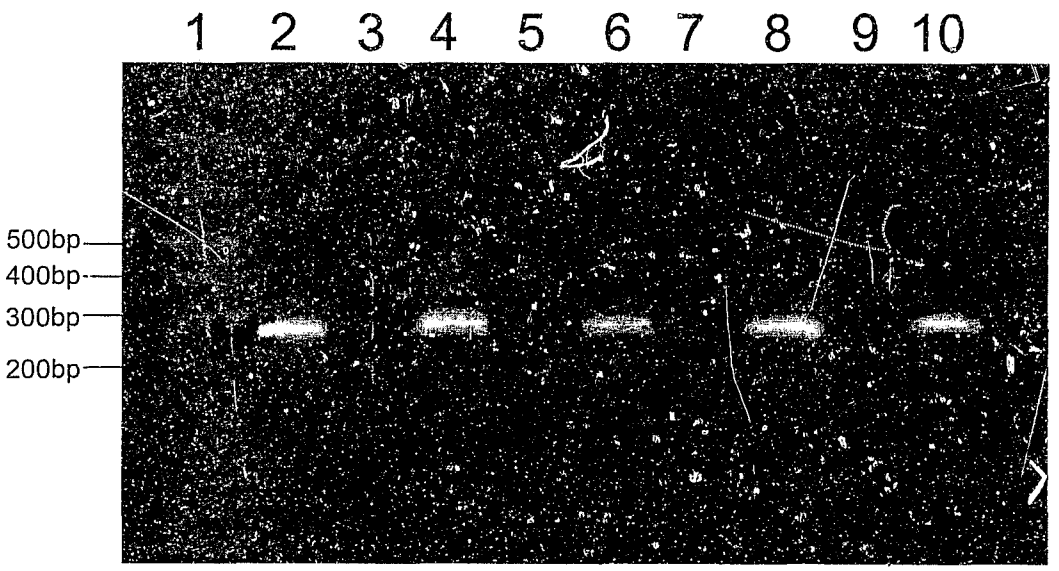


Figure 8 Detection of 260bp PCR product on 2% agarose gel.
Lane 1: low molecular weight marker
Lane 2: positive control (serum positive for HBsAg, HBeAg and HBV DNA)
Lane 3: negative control (serum from healthy black individual lacking all HBV markers)
Lanes 4, 6, 8, 10: patients positive for HBV DNA
Lanes 5, 7, 9: water controls

3.2 Sequencing analysis

Sequence analysis was only possible in samples with an amplifiable X gene without deletions or insertions. HBV DNA amplified from both serum samples and liver tissue was successfully sequenced in the region nt1683 to nt1849, which includes part of the enhancer II (nt1636 - nt1741) and the BCP (nt1742 - nt1849). Furthermore this region is translated into the carboxyl-terminus of the X protein and the amino terminus of the HBeAg precursor.

3.2.1 Enhancer II sequences

The sequence of the enhancer II region (nt1683 - nt1741), was found to be reasonably well conserved when aligned to sequence EMBL X70185 (subtype *adw2*), (Preisler-Adams *et al.*, 1993), (figures 15 – 20, appendix A), and was not affected by either the HBeAg/anti-HBe status or the disease severity *viz.* ASC vs. HCC.

3.2.2 BCP sequences

In total 42 different nucleotide sequence patterns of the BCP region (nt1742 - nt1849), when aligned to EMBL X70185, were detected in HBV DNA extracted from sera of ASCs and HCC patients and tumorous and non-tumorous liver samples, (Figures 9 to 12). Highlighted sequences were unique to a particular patient population or tissue sample.

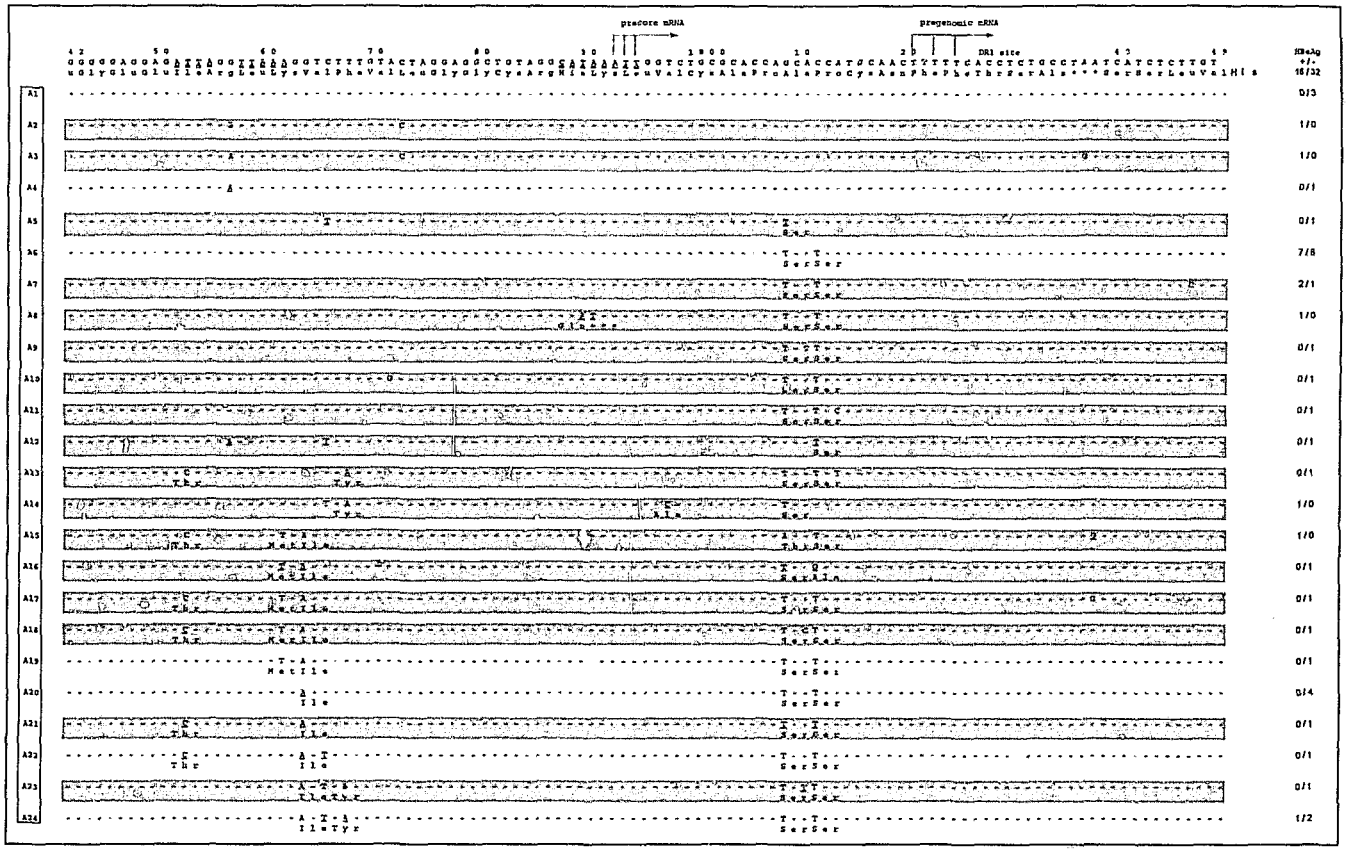


Figure 9 Nucleotide and deduced amino acid sequence of the BCP (1742 - 1849) of HBV DNA isolated from ASCs aligned to sequence EMBL X70185 (shown in full). TA-rich regions are underlined with a double line. Twenty-four different sequences were found in 47 ASCs. Highlighted sequences were found only in sera of ASCs. Numbers on the right are the number found in HBeAg-positive (+) and HBeAg-negative (-) carriers. Substitutions that occurred together with wildtype nucleotides are underlined. *** indicates a nonsense mutation.

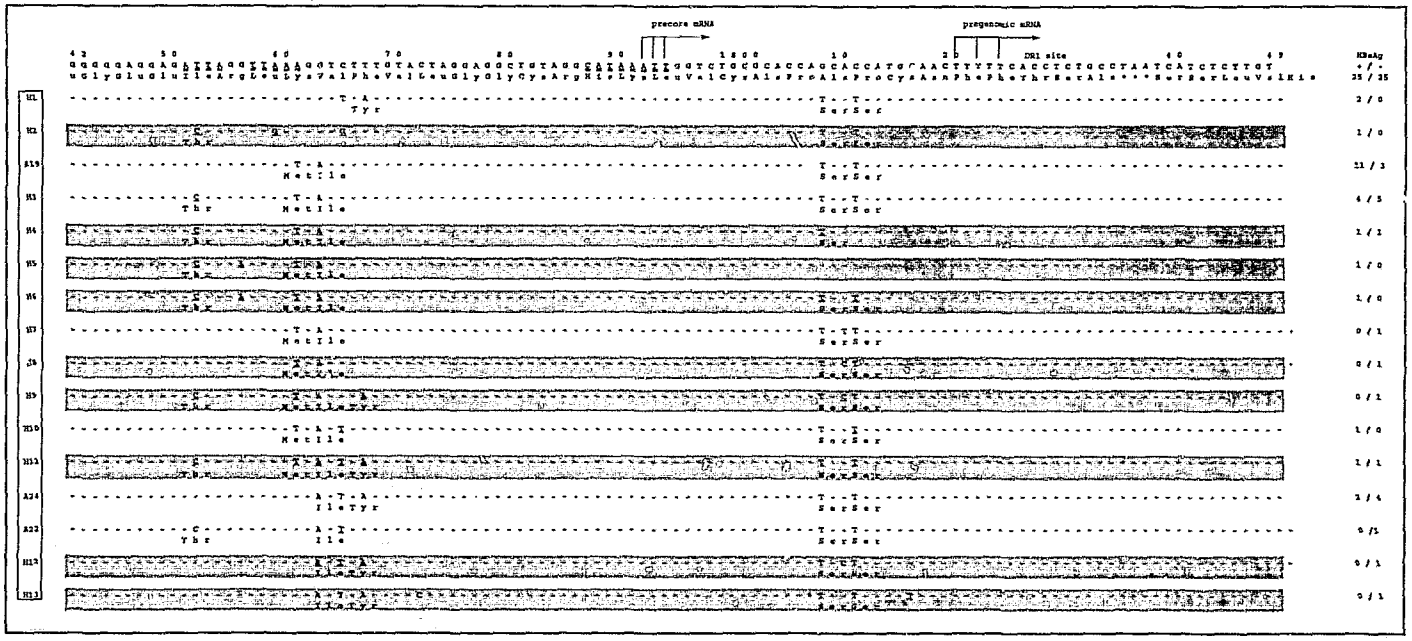


Figure 10 Nucleotide and deduced amino acid sequence of the BCP (1742 - 1849) of HBV DNA isolated from HCCpatients aligned to sequence EMBL X70185 (shown in full). TA-rich regions are underlined with a double line. Seventeen different sequences were found in 50 HCC patients. Highlighted sequences were found only in sera of HCC patients. Numbers on the right are the number found in HBeAg-positive (+) and HBeAg-negative (-) carriers. Substitutions that occurred together with wildtype nucleotides are underlined.

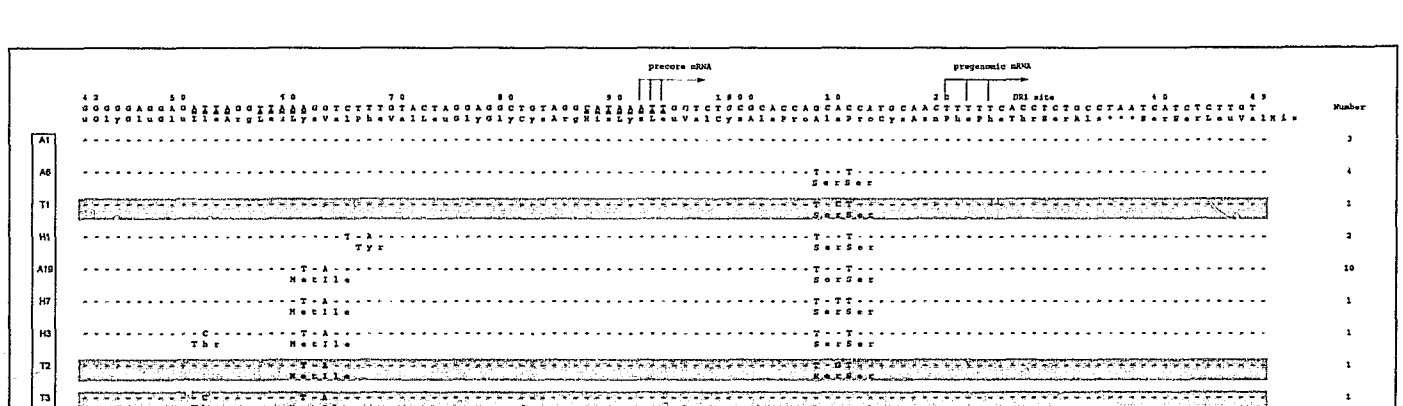


Figure 11 Nucleotide and deduced amino acid sequence of the BCP (1742 - 1849) of HBV DNA isolated from tumorous liver tissue aligned to sequence EMBL X70185 (shown in full). TA-rich regions are underlined with a double line. Thirteen different sequences were found in 29 tumorous liver tissue samples. Highlighted sequences were found only in tumorous liver tissues. Substitutions that occurred together with wildtype nucleotides are underlined.

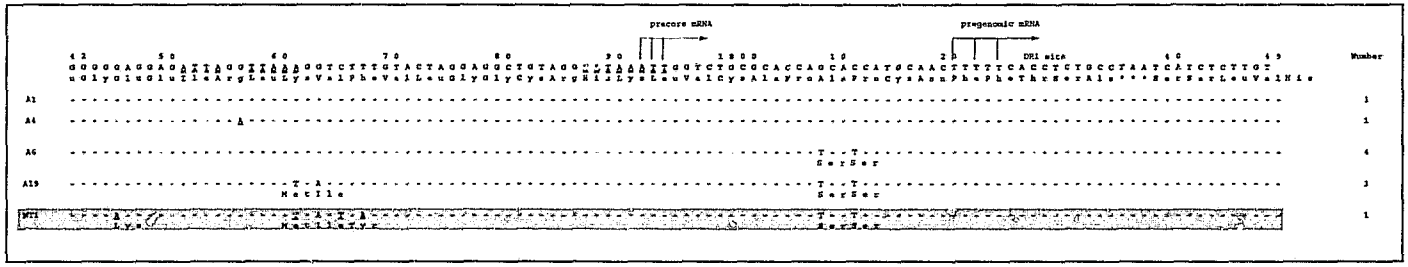


Figure 12 Nucleotide and deduced amino acid sequence of the BCP (1742 - 1849) of HBV DNA isolated from non-tumorous liver tissue aligned to sequence EMBL X70185 (shown in full). TA-rich regions are underlined with a double line. Five different sequences were found in 10 non-tumorous liver tissue samples. Highlighted sequences were found only in non-tumorous liver tissue. Substitutions that occurred together with wildtype nucleotides are underlined.

In HBV from serum samples, missense mutations were found in 40% of the ASC patients whereas 80% of HCC-derived sequences had missense mutations. Seventy one percent (71%) of tumorous tissue as opposed to 44% on the non-tumorous tissues samples had missense mutations, (figures 21-26, appendix A).

Table 6 compares nucleotide and amino acid sequence divergence in BCP and precore regions of ASCs and HCC patients (precore sequence results from previous study, Kramvis *et al.*, 1997, 1998a). Both nucleotide and amino acid sequences were better conserved in the precore region compared to the BCP. Percent nucleotide and amino acid sequence divergence was significantly higher in the BCP compared to the precore region ($p<0.0001$). In the BCP both nucleotide and amino acid sequence divergence were significantly higher in virus extracted from HCC patients compared to ASCs ($p < 0.0001$).

In 5 patients the BCP sequences of HBV DNA were found to be identical in both tumorous and non-tumorous tissues. In another 5 patients, tumorous tissues had sequences different to those found in the corresponding non-tumorous tissues. The majority (4/5) of these latter tumor-derived sequences had missense mutations whereas those derived from the non-tumorous tissues had wild-type sequences (table 7, figure 11 and 12). However, there was no significant difference between nucleotide and amino acid divergence in tumorous and non-tumorous tissue. Both tumorous and non-tumorous liver tissue has a nucleotide divergence of 3% whereas tumorous tissue has an amino acid divergence of 8.6% and non-tumours tissue of 7%.

Table 6 Nucleotide and amino acid sequence divergence in the BCP and precore regions of HBV isolates from sera of ASCs and HCC patients.

	BASIC CORE PROMOTER			PRECORE REGION		
		% nucleotide divergence	% amino acid divergence		% nucleotide divergence	% amino acid divergence
	#	Mean ±S.D.	mean ±S.D.	#	mean ±S.D.	mean ±S.D.
ASC						
TOTAL	47	2.7 ± 1.5*a	6.8 ± 3.9*b	57	1.5 ± 1.3	2.2 ± 2.6
HBeAg+ve	15	2.4 ± 1.3	6.3 ± 3.6	16	1.5 ± 2.2	1.5 ± 2.2
HbeAg-ve	32	2.8 ± 1.6	7.0 ± 4.0	41	1.6 ± 1.3	2.3 ± 2.7
HCC						
TOTAL	50	4.0 ± 1.5*a	10.7 ± 4.0*b	61	1.5 ± 1.3	2.2 ± 2.7
HBeAg+ve	25	4.2 ± 2.0	11.5 ± 2.0	23	1.5 ± 1.3	1.5 ± 20
HbeAg-ve	25	3.8 ± 2.0	9.9 ± 5.3	38	1.5 ± 1.3	2.7 ± 3.0
TOTAL	97	3.3 ± 1.6	8.8 ± 4.4	118	1.5 ± 1.3	2.2 ± 2.6
HBeAg+ve	40	3.5 ± 1.3	9.5 ± 3.7	39	1.5 ± 1.2	1.5 ± 2.0
HbeAg-ve	57	3.2 ± 1.8	8.3 ± 4.8	80	1.5 ± 1.3	2.5 ± 2.8

These results are derived from previous studies [Kramvis et al, 1997, 1998a]
* These results are statistically different using the Student t test.
a: $p < 0.0001$ comparing nucleotide divergence in BCP of HBV isolates from ASC and HCC patients.
b: $p < 0.0001$ comparing amino acid divergence in BCP of HBV isolates from ASC and HCC patients.

Table 7 Sequence analysis of HBV DNA extracted from tumorous and non-tumorous liver samples.

Group	Status	#	Sequence analysis	
			Tumor	Non-tumor
A	HBsAg+ve/HBeAg+ve	L1	Missense (A19)[a]	Missense (A19)[a]
		L2	Wild-type (A6)	Wild-type (A6)
B	HBsAg+ve/HBeAg-ve	L3	Wild-type (A6)	Wild-type (A6)
		L4	Missense (A19)[a]	Missense (A19)[a]
		L5	Missense (H7)[a]	Wild-type (A6)
		L6	Missense (A20)[b]	Wild-type (A4)
		L7	Missense (A19)[a]	Wild-type (A6)
		L.	Missense (H3)[a]	NA
		L9	Missense (H1)	NA
		L10	Missense (A19)[a]	NA
		L11	Missense (T4)[a]	NA
		L12	Wild-type (T1)	NA
		L13	Missense (H10)[a]	NA
		L14	Missense (A24)[b]	NA
		L15	Wild-type (A1)	NA
		L16	Wild-type (A1)	NA
		L17	Wild-type (A1)	NA
		L18	Missense (A19)[a]	NA
		L19	Missense (A24)[b]	NA
		L20	Missense (T2)[a]	NA
		L21	Missense (A19)[a]	NA
		L22	Missense (H1)	NA
C	Anti-HBs+ve/anti-HBc+ve	L23	Missense (A19)[a]	Missense (A19)[a]
		L24	Missense (A19)[a]	Wild-type (A1)
		L25	Wild-type (A6)	Missense (NT1)[a]
		L26	Wild-type (A6)	NA
		L27	Missense (A19)[a]	NA
		L28	Missense (T3)[a]	NA

Numbers in brackets refer to sequence numbers in figures 9-12.
a: with $1762^{T}1764^{A}$ **b:** with 1764^{A} **NA:** tissue not available

The BCP sequences obtained from both sera and tissues were characterised by 2 very well conserved regions: nt1770 - nt1808 and nt1813 - nt1849; and 2 hyper variable regions: nt1753 - nt1769 and nt1809 - nt1812. In a GenBank search sequence M57663 (subtype *adw;* isolated from a Philippino) (Estacio *et al.*, 1988) was found to have the same mutations at 1809 and 1812. These missense mutations, 1809 (G T; alanine to serine) and 1812 (C T; proline to serine) may be characteristic of the virus found in southern African blacks because they were detected in 80% of the sequences.

The most common changes in the first hyper variable region nt1753 - nt1769 were the missense mutations at 1762 (A T; lysine to methionine) and 1764 (G A; valine to isoleucine), (figure 13). The 1762^{T} was never found to occur in the absence of 1764^{A} whereas 1764^{A} did occur alone, (figure 13). Table 8 compares the frequency of these single and double BCP mutations in the different patient populations and in the HBeAg-positive and HBeAg-negative serum samples. There was no significant difference in the incidence of the double 1762^{T} 1764^{A} mutant in HBeAg-negative patients when compared to HBeAg-positive patients. However, when HBeAg titers were compared semi-quantitatively in HBeAg-positive ASCs and HCC patients, with wild-type precore region, (see appendix A), the levels were significantly lower in patients carrying the virus with the 1762^{T} 1764^{A} double mutants, (figure 14) i.e., 3.0 in the mutant versus 7.8 in the wild-type ($p < 0.0001$). This equates to a 60% reduction in HBeAg expression. The frequency of the double 1762^{T} 1764^{A} mutant was significantly higher in HCC patients when compared to ASCs (table 8) ($p<0.0001$). Sequence A19 was the sequence most commonly found in sera and tissue of HCC patients, (figures 10-12) and only in one ASC, (figure 9). Moreover,

sequence H3, which in addition to the $1762^{T}1764^{A}$ has a mutation at position 1753, was only found in sera and tissue of HCC patients and not ASCs.

Table 8 Frequency of BCP mutations in HBV isolates from ASC and HCC patients.

Sample	Patients	#	1762^{T} 1764^{A} mutant	1764^{A} mutant	TOTAL
Sera					
ASC		47	5 (11%)*a	10(21%)	15 (32%)*b
HBeAg+ve		15	1 (7%)	1 (7%)	2 (14%)
HBeAg-ve		32	4 (13%)	9 (28%)	13 (41%)
HCC		50	33 (66%)*a	9 (18%)	42 (84%)*b
HBeAg+ve		25	20 (80%)	2 (8%)	22 (88%)
HBeAg-ve		25	13 (52%)	7 (28%)	20 (80%)
Total		97	38 (39%)	19 (20%)	57 (59%)
HBeAg+ve		40	21 (53%)	3 (8%)	24 (61%)
HBeAg-ve		57	17 (30%)	16 (28%)	33 (58%)
Tissues					
Tumorous		28	15 (54%)	3 (11%)	18 (65%)
Nontumorous		10	4 (40%)	0 (0%)	4 (40%)

* These results are statistically different using the c^2 test
a: $p < 0,0001$ comparing the frequency of $1762^{T}1764^{A}$ mutant in HCC vs. ASC
b: $p < 0,0001$ comparing the frequency of $1762^{T}1764^{A}$ and 1764^{A} mutant in HCC vs. ASC.

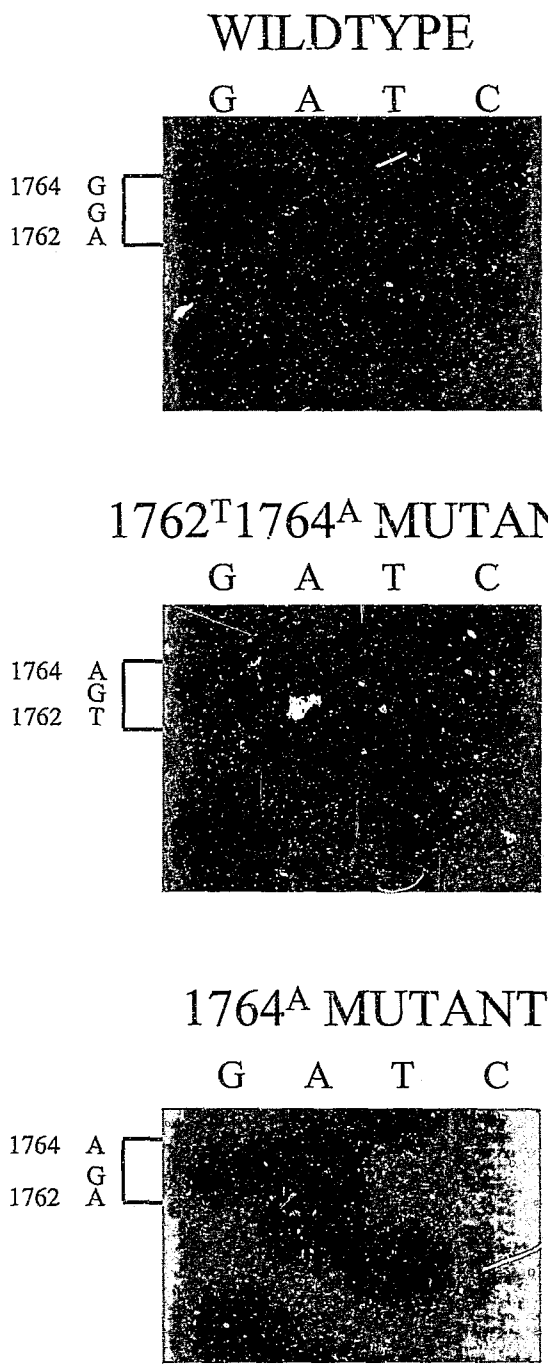


Figure 13 Autoradiographs demonstrating the wildtype $1762^{A}1764^{G}$ sequence, the $1762^{T}1764^{A}$ double mutant sequence and the 1764^{A} mutation found together with the wildtype 1762^{A} sequence.

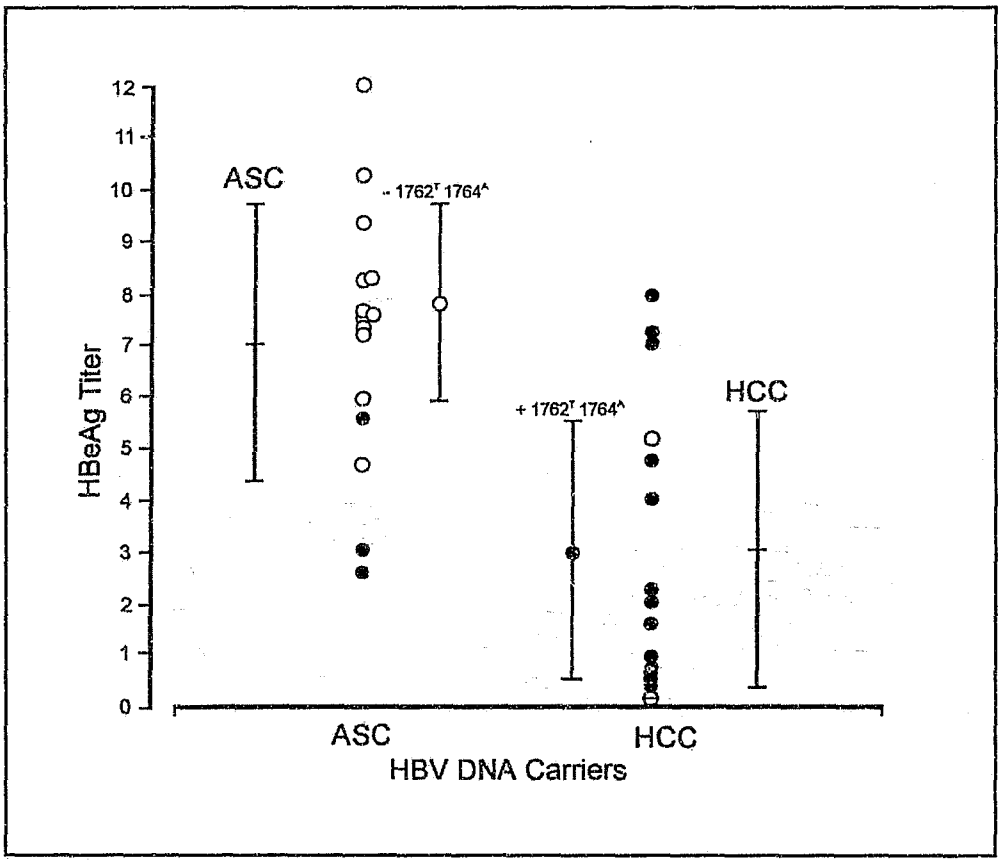


Figure 14 Comparison of HBeAg titres between HBeAg-positive and HCC patients with and without 1762^T1764^A.

HBeAg titres as determined semi-quantitatively by radioimmunoassay of serum samples. A level of 1.0 corresponds to 1,000cpm. Black and white points are for samples with and without 1762^T1764^A, respectively. The white point with slash is a sample with 1764^A without 1762. The serum HBeAg titres were significantly lower in patients with 1762^T1764^A than those without ($p<0.0001$). The difference in HBeAg titers between HBeAg-positive ASCs and HCC patients were also significantly different ($p<0.0001$). The mean and standard deviation from each group are shown.

Chapter 4: DISCUSSION

HBV has a small compact genome with four overlapping regions, viz. polymerase, surface, X and precore/core regions (Gerber *et al.*, 1985; Tiollais *et al.* 1985, 1988). The region sequenced, nt1685 to nt1849, is fairly compact and complex containing the BCP, part of the enhancer II, and encodes for the carboxyl-terminus of the X protein.

The compact nature and complexity of this portion of the genome has resulted in a 164-nucleotide region that has a dual function (Yaginuma *et al.*, 1987; Yuh *et al.*, 1992; Chen *et al.*, 1995a). Firstly, the BCP and enhancer II are important elements in the control of transcription of both the precore mRNA and pregenomic mRNA (Yuh *et al.*, 1992). The precore mRNA is translated into the precore/core protein that is post-translationally processed to produce the HBeAg, an important marker of infectivity and viral replication. The pregenomic mRNA is translated into the core and polymerase proteins, while a small fraction is encapsidated into core particles and reverse transcribed into the viral genome (Bonino *et al.*, 1991; Schaller *et al.*, 1991; Yuh *et al.* 1992; Okamoto *et al.*, 1994; Chen *et al.* 1995a; Sato *et al.*, 1995; Baumert *et al.*, 1996; Scaglioni *et al.*, 1997a;). The second important function of this region is encoding for part of the X protein, which has transactivation functions. The DR1, which is important for primer binding during genome replication, is also found within the region sequenced (Kidd-Ljunggren *et al.*, 1997).

Close analysis of this region was therefore important because mutations may affect HBeAg production and/or viral replication and/or the amino acid sequence of the X protein.

Nucleotide and amino acid sequence divergences were both significantly lower in the precore region than in the BCP (table 6). This was true for both the ASCs and patients with HCC. This supports previous observations that inferred that BCP changes appear earlier than precore changes (Chang *et al.*, 1999; Shindo *et al.*, 1999). Also, it lends support to recent reports that suggest that BCP mutations may develop more frequently in patients with HBV genotypes that prevent the development of the precore stop codon mutation (Lindh *et al.*, 1998; Chan *et al.*, 1999), as is the case in the southern African black population (Kramvis *et al.*, 1997; 1998a).

Interestingly, the nucleotide and amino acid sequence divergences were similar in ASCs and patients with HCC in the precore region, but differed significantly in the BCP. Both the nucleotide and amino acid sequence divergences were significantly higher in the patients with HCC ($p < 0.001$). 80% of HCC-derived sequences had missense mutations compared to 40% in ASCs. Since the nucleotide and amino acid substitution rates in the X and core regions have been reported to generally be similar, the higher nucleotide and amino acid sequence divergence in HCC patients suggests a selection of virus with missense mutations in this region (Orito *et al.*, 1989). When tumor and non-tumor sequence divergences were compared, no significant differences were noted. Hsia *et al.* (1996) found the same in tumor and adjacent non-tumor tissues from patients with HCC from Qidong and Beijing. In our study, however, the number of non-tumor tissues was too few to draw any firm conclusions.

Two highly conserved regions were identified within the sequenced region, *viz.* nt1770 to nt1808 and nt1813 to nt1849. This is in agreement with other studies

(Okamoto *et al.*, 1994; Laskus *et al.*, 1995; Kidd-Ljunggren *et al.*, 1995, 1997; Honda *et al.*, 1999). It is not surprising that these regions are so well conserved because they are crucial in regulating transcription and replication of HBV.

In the region from nt1770 to nt1808 mutations were found in only 2/136 sequences and these occurred as mixed populations with the wildtype sequence. The wildtype sequence is thought to have a compensatory effect overcoming the potentially lethal effect of the mutation and thus allowing for normal viral function. Sequences corresponding to the initiator sequence of the precore promoter (nt1788 - nt1791) and TATA sequence of the pregenomic promoter (nt1788 - nt1794) have been mapped to this region (Chen *et al.*, 1995a; Yu *et al.*, 1996). Thus, changes in this region could drastically alter transcription levels of precore mRNA and pregenomic mRNA. Additionally, sequences in this region correspond to the Kunitz domain of serine protease inhibitors that have been shown to be essential for the transactivating function of the X protein (Arii *et al.*, 1992; Koike *et al.*, 1995).

The second well conserved region (nt1813 to nt1849) codes for the carboxyl-terminus of the X protein. Even though this region is thought not to be essential for transactivation (Ritter *et al.*, 1991; Arii *et al.*, 1992; Runkel *et al.*, 1993), its intactness is essential to maintain the X protein's overall secondary structure that allows for its proper and efficient functioning. The initiator sequence for the pregenomic promoter (nt1817 - nt1821) is also located in this region, as is DR1. Thus, failure to conserve this sequence would result in no viral replication.

In this study, mutations 1809 and 1812 were found in 80% of all sequences. These mutations have previously been reported by Estacio *et al.* (1988) in a Philippino man,

in 1 out of 29 HBV stains from 14 different countries and by Laskus *et al.* (1994b) in chronic hepatitis patients after orthotopic liver transplantation. As a result of the high incidence of these mutations in the southern African isolates it is likely that these mutations represent wildtype sequences in this population rather than mutations. These changes disrupt the Watson-Crick base pairs in the secondary structure of the pregenomic RNA around DR1, but seemingly do not interfere with viral replication and/or do not seem to affect the function of the X protein (Kidd *et al.*, 1996). These mutations result in missense mutations converting the hydrophobic, larger alanine and proline to the smaller and polar serine amino acids. It is possible that 1809 or 1812, or both, affect the translation of HBeAg because these mutations, particularly 1812, change the optimal sequence for initiation by eukaryotic ribosomes (reviewed by Kramvis *et al.*, 1999).

The most common missense mutations occurring in the BCP in southern African isolates were the 1762^{T} and 1764^{A} in tandem and 1764^{A} alone. 1762^{T} alone was not found. This is in agreement with the HBeAg seroconversion longitudinal study carried out by Chan *et al.* (1999). In their study they found that in a few cases 1764^{A} first arose as an intermediate step, while 1762^{T} remained wildtype. In no cases did 1762^{T} arise on its own (Chan *et al.*, 1999). This had also previously been documented by Kidd-Ljunggren *et al.* (1997). However, contradicting our results, Erhardt *et al.* (2000) and Lindh *et al.* (1999) recently reported that 1762 A to T often develops during HBe seroconversion, particularly in strains without precore mutants that prevent HBeAg production. Previous studies rarely report either of these mutations to occur on their own. They commonly occur together and have been found to occur in ASCs (Takahashi *et al.*, 1995, 1999; Kurosaki *et al.*, 1996; Lindh *et al.*, 1999), patients with self-limiting or severe acute hepatitis (Aritomi *et al.*, 1998), patients with chronic

hepatitis (Tong *et al.*, 1990; Horikita *et al.*, 1994; Laskus *et al.*, 1995; Takahashi *et al.*, 1995, 1999; Buckwold *et al.*, 1996; Kanai *et al.*, 1996; Kim *et al.*, 1999; Kurosaki *et al.*, 1996; Sterneck *et al.*, 1996; Hou *et al.*, 1999), patients with fulminant hepatitis (Ogata *et al.*, 1993; Hasagawa *et al.*, 1994; Horikita *et al.*, 1994; Kaneko *et al.*, 1995; Laskus *et al.*, 1995; Sato *et al.*, 1995; McMillan *et al.*, 1996; Aritomi *et al.*, 1998) and patients with HCC. (Loncarevic *et al.*, 1990; Blum *et al.*, 1991; Takahashi *et al.*, 1995, 1998, 1999; Hsia *et al.*, 1996).

It was found that the incidence of 1764^{A} on its own was similar in ASC and patients with HCC. The prevalence of the double missense mutation, 1762^{T} along with 1764^{A} was significantly ($p<0.001$) higher in patients with HCC when compared to ASCs. This is in agreement with study of Fang *et al.* (1998). The 1762^{T} mutation was found in 68% of HCC isolates and 1764^{A} mutation in 80%, in comparison to 13.5% and 38% in ASC isolates, respectively. This considerable step-up between the ASCs and patients with HCC suggests that these mutations may play a role in HBV-induced hepatocarcinogenesis because in black Africans it is usually the ASCs in whom HCC develops. In 1995 Laskus *et al.* showed that $1762^{T}1764^{A}$ was common in cirrhotic patients with end-stage liver disease. They speculated that these mutations may accumulate over time and that in long-term infections these variants may cause more serious liver disease (Laskus *et al.*, 1995). Aritomi *et al.* (1998) also found that the precore 1896 stop mutation and $1762^{T}1764^{A}$ mutations increased proportionally with severity of disease, with fulminant hepatitis patients having a higher frequency of these mutations when compared to patients with acute hepatitis. Hou *et al.* (1999) also noted that the double mutation was significantly more common in Japanese patients with chronic hepatitis than ASC. In contrast, McMillian *et al.* (1996) found no association between the $1762^{T}1764^{A}$ mutations and the severity of disease in

patients who had severe recurrent disease post-transplantation. The incidence of 1762^{T} and 1764^{A} mutations in Japanese patients was not significantly different from those in the southern African isolates and therefore may suggest a similar if not identical mechanism/s leading to the progression of HCC (Takahashi *et el.,* 1998).

In our study no association was found between $1762^{T}1764^{A}$ and HBeAg negativity. This is in agreement with studies carried out by Laskus *et al.* (1995), Takahashi *et al.* (1995), McMillian *et al.* (1996), Nagasaka *et al.* (1998) and Lindh *et al.* (1999) but contrasts with the results of Okamoto *et al.* (1994), Nashizono *et al.* (1995), Sato *et al.* (1995), Kidd-Ljunggren *et al.* (1996, 1997), Kurosaki *et al.* (1996) and Hou *et al.* (1999). Comparing HBeAg-titers of the HBeAg-positive sera with $1762^{T}1764^{A}$ with sera with wildtype virus, revealed that in patients carrying the mutant virus, the HBeAg titers were reduced by 60%. This supports the findings of Takahashi *et al.* (1995), Buckwold *et al.* (1996), Kurosaki *et al.* (1996), and Erhardt *et al.* (2000), indicating that the double mutation suppresses HBeAg-production, rather than abolishing its production altogether. These results have been confirmed in *in vitro* systems (Buckwold *et al.*, 1996, 1997; Moriyama *et al.*, 1996; Günther *et al.*, 1998). In the studies carried out by Buckwold *et al.* (1996, 1997b) and Moriyama *et al.* (1996), the $1762^{T}1764^{A}$ mutations resulted in reduced transcription levels of the precore mRNA, and thus decreased HBeAg synthesis.

Functional studies by Yu *et al.* (1996) have shown that precore mRNA and pregenomic mRNA are under the control of two distinct promoters. The 1762^{T} mutation falls within the TATA-like box of the precore promoter controlling the transcription of precore mRNA (Sato *et al.*, 1995). In eukaryotic promoters, point mutations in the TATA sequence motif have been shown to drastically decrease

specific *in vitro* transcription. Thus, if this concept is extended to viral promoters, it may be speculated that the TTAAA → TTAAT change in the TATA-like box reduces the efficiency of the precore promoter without affecting its precision of initiation, resulting in decreased HBeAg production, rather than its complete abolishment, (figure 14), (Okamoto *et al.*, 1994; Chen *et al.*,1995a; Laskus *et al.*, 1995; Kurosaki *et al.*, 1996). *In vitro* studies conducted by Buckwold *et al.* (1997) support this hypothesis. They found that 1764^{A} on its own has no effect on precore mRNA transcription, whereas 1762^{T} on its own gives the same HBeAg-suppressive phenotype as the $1762^{T}1764^{A}$ double mutation. Moriyama *et al.* (1997) concluded from their deletion *in vitro* studies that nt1757 to nt1762 are important for efficient initiation of the precore mRNA, thus providing indirect evidence that nt1762 is important for proper BCP functioning. A longitudinal study carried out by Chan *et al.* (1999), following sequence changes in patients seroconverting from HBeAg-positive to HBeAg-negative, provides further indirect evidence for the HBeAg suppressed phenotype. The $1762^{T}1764^{A}$ missense mutations have been shown to arise before the stop codon mutation 1896, thus reflecting that the BCP mutations are inefficient in completely preventing HBeAg production (Lindh *et al.*, 1998; Chan *et al.*, 1999).

In vitro studies have generated controversial results with regards to the effects of $1762^{T}1764^{A}$ on viral replication. Using a reporter gene under a mutated promoter, Nishizono *et al.* (1995) failed to detect a significant difference in promoter activity when comparing mutant and wildtype HBV. In transfection studies $1762^{T}1764^{A}$ suppressed precore mRNA transcription and enhanced viral replication (Buckwold *et al.*, 1996, 1997; Moriyama *et al.*, 1996, 1997). Buckwold *et al.* (1996, 1997) found, from their transfection studies, that a liver specific-enriched transcription factor (which they identified as the chicken ovalbumin upstream promoter-transcription factor

(COUP-TF) or a protein closely related to it) binds to nt1756 to nt1766. This site directly covers the $1762^{T}1764^{A}$ mutation. The 1762^{T} mutation only or the $1762^{T}1764^{A}$ double mutation together, were both shown to prevent the binding of this transcription factor. The result was a reduction of the precore mRNA transcription to 1/3 of the wildtype level. Furthermore, replication of the mutant virus was significantly enhanced relative to the wildtype vir ., possibly because the reduction in the e antigen allowed for enhanced packaging efficiency of the pregenomic RNA. It has been speculated that the small amounts of e antigen that are released into the cytosol are likely to suppress HBV progeny production immediately prior to the encapsidation step in a dominant-negative regulatory manner, since this protein is structurally similar to the core protein. The $1762^{T}1764^{A}$ double mutation did not affect the transcription of any of the other HBV genes (Buckwold *et al.*, 1996, 1997). The transfection studies of Moriyama *et al.* (1996, 1997) and Buckwold *et al.* (1996, 1997) were in general agreement. However, Moriyama *et al.* (1996, 1997) attributed the increase of viral replication to an increase in pregenomic RNA transcription rather than enhanced encapsidation suggested by Buckwold *et al.* (1996, 1997). In contrast Günther *et al.* (1998) found reduced precore mRNA transcription only and no increased viral replication in transfection studies using HuH7 cells. The fact that we and others have detected these mutants in viral isolates from sera of ASC and HCC patients, which have low HBV DNA titers (based on the fact that HBV DNA could be detected only by using nested PCR), argues against these mutations causing increased viral replication. Further support for this view is provided by the finding that DNA polymerase levels were significantly lower in patients with the double mutant virus (Kurosaki *et al.*, 1996). Thus, the prevalence of the $1762^{T}1764^{A}$ mutant virus over wildtype virus in HCC patients appears not to result from increased viral replication but may be secondary to decreased HBeAg expression.

Comparison of *in vivo* and *in vitro* results must be exercised with extreme caution because *in vitro* systems do not allow for the testing of certain *in vivo* conditions; for example: the interactions between the virus and the immune system, the different ratios of mutant to wildtype virus during natural infection and the effect of different genotypes. Subtle differences in factors like these may therefore cause variable results, as is often seen in similar studies.

Besides the $1762^{T}1764^{A}$ mutations other mutations were found in the BCP of ASC and patients with HCC, in varying proportions. These include a T to C missense mutation at nt1753, a silent C to T mutation at nt1766 and a T to A missense mutation at nt1768. All these mutations cluster around the $1762^{T}1764^{A}$ double mutation, a region previously noted by Kidd-Ljunggren *et al.* (1997) as being hypervariable.

The 1753 T to C missense mutation was always found together with 1762 and/or 1764 mutation/s. Its incidence was similar in both HBeAg-positive and HBeAg-negative ASC and HCC patients. This contradicts the results that found 1753 to occur more frequently in HBeAg-negative patients (Nagasaka *et al.*, 1998). In our study we found that 1753 occurred at a significantly higher rate ($p=0.015$ with Yates correction) in HCC patients when compared to the rate seen in ASCs. This was to be expected since 1753 was only found coupled to 1762 and/or 1764 mutation/s and these were found at a significantly higher rate in HCC patients. An *in vitro* study carried out by Günther *et al.* (1998) studying the effects of these three linked mutations found that they did not enhance viral replication, they had no effect on the level of pregenomic mRNA, they decreased precore mRNA and HBeAg and caused no change to the HBsAg and polymerase levels. Kidd *et al.* (1996) proposed that the $1762^{T}1764^{A}$

double mutation and other mutations at nt1751 to nt1757 are likely to cause structural changes in the secondary structure of the pregenomic RNA (nt1742 – nt1847). They speculated that these changes would result in enhanced viral replication. However, the results of Günther *et al.* (1998) *in vitro* study refute this proposal.

The 1766 and 1768 mutations were more common in HCC patients than ASC, but these differences were not statistically significant. They were found in both HBeAg-positive and HBeAg-negative ASCs and HCC patients, thus seemingly not interfering with HBeAg production. *In vitro* studies of these mutations found that these mutations resulted in enhanced encapsidation and replication, caused a minor increase in transcription of pregenomic mRNA and increase HBeAg expression *in vitro*. Transfection studies looking at the combined effect of the 1762, 1764, 1766 and 1768 mutations in tandem found that there was a decrease in the levels of precore mRNA (and thus HBeAg), the pregenomic mRNA synthesis was not affected and viral replication was only moderately enhanced (Scaglioni *et al.*, 1997b). Mutations at 1764 and 1766 or 1766 and 1768 have been reported in children with multiple anti-HBe/HBeAg reactivations. In both cases these mutation combinations resulted in enhanced core promoter activity likely due to novel or altered binding sites for liver enriched or ubiquitous transcription factors (Gerner *et al.*, 1999).

A number of liver-enriched and ubiquitous cellular transcription factors have been shown to bind to the BCP and to be important in determining the level of transcription from HBV promoters, (table 4), (Johnson *et al.*, 1995). Often the sequence to which one particular transcription factor binds to is the same or overlaps with the sequence to which another transcription factor may bind. Mutations may cause transcription

factor binding to be abolished, altered (either increased or decreased) and/or may even create a new transcription factor binding site (Li *et al.*, 1995; Gunther *et al.*, 1996; Pult *et al.*, 1997; Raney *et al.*, 1997; Buckwold *et al.*, 1999). The CCAAT/enhancer binding protein (C/EBP) (López-Cabrera *et al.*, 1990), several members of the nuclear receptor superfamily (viz. COUP-TF1, HNF4, TR2, PPAR-α-RXR-α, PPAR-γ-RXRα) (Buckwold *et al.*, 1997; Raney *et al.*, 1997; Yu *et al.*, 1997), HNF4 (Guo *et al.*, 1993; Raney *et al.*, 1997), rat liver nuclear extract (RLNE) (López-Cabrera, *et al.*, 1990), liver-enriched protein factor (LEF) (Buckwold *et al.*, 1996), TBP (Günther *et al.*, 1996) and other unidentified transcription factors (Zhang *et al.*, 1997) have all been mapped to bind the region within which the $1762^{T}1764^{A}$ mutations occur. These changes may result in the temporal or differential regulation of transcription of the precore and pregenomic promoter (Jonhson *et al.*, 1995; Kurosaki *et al.*, 1996; Raney *et al.*, 1997; Yu *et al.* 1997). From this study however, no conclusions can be drawn as to how these mutations may or may not affect transcription factor binding and thus promoter functioning.

From this and other studies it is obvious that mutations in the BCP are more prevalent in HCC patients relative to ASC. *In vivo* and *in vitro* studies have shown that these mutations generally suppress HBeAg synthesis and this may contribute to hepatocarcinogenesis. However, the exact mechanisms involved are unknown.

HBeAg shares epitopes with HBcAg and this has been postulated to induce immune tolerance against itself or HBcAg or both antigens (Milich *et al.*, 1990; Carman *et al.*, 1993). It reduces the immune pressure on HBcAg by diverting the immune effector cells away from the infected hepatocytes, allowing for prolonged viral survival (Carman *et al.*, 1992, 1993; Thomas *et al.*, 1994; Carman, 1995). However, in the

presence of reduced or absent serum HBeAg, HBcAg may be targeted directly by both the cellular and humoral immune systems (Milich *et al.*, 1988) leading to necrosis of hepatocytes and liver damage (Brunetto *et al.*, 1991; Kaneko *et al.*, 1995). HBcAg elicits a significantly more vigorous antibody response *in vivo* than HBeAg (Milich *et al.* 1988). Liver damage is an important contributing factor in the development of HBV-related HCC (Kew, 1996). This offers one possible mechanism by which $1762^{T}1764^{A}$ and other BCP mutations, that reduce HBeAg synthesis, may contribute to hepatocarcinogenesis.

Missense mutations in the BCP could affect the structure of the X protein. $1762^{T}1764^{A}$ are nonsynonymous and convert amino acids 130 and 131 of the overlapping X coding region from lysine to methionine (1762; A→T, AAG→ ATC) and valine to isoleucine (1764; G→A, GTC→ATC), respectively. Lysine is a charged amino acid, whereas methionine is hydrophobic, and this alteration could be the reason why the 1762 mutation is rarely found alone. On the other hand, because both valine and isoleucine are hydrophobic the 1764^{A} mutation on its own is better tolerated. A study by Li *et al.* (1999) demonstrated that the two-codon change in the X protein is able to suppress both pre-C mRNA and pregenomic RNA synthesis. However, $1762^{T}1764^{A}$ double mutant creates a HNF1 site. Thus, this partially counteracts the suppressive effect and restores pregenomic RNA, but not precore mRNA levels (Li *et al.*, 1999).

The amino acid changes caused by the missense mutations clustered around $1762^{T}1764^{A}$ and 1809/1812 may alter the structure of the X protein. Structural changes to the protein may alter its function. Functional changes could result in it interacting in a modified manner with cellular and viral genes and/or promoters. This

could lead to changes occurring in essential pathways, thereby contribute to hepatocarcinogenesis.

Chapter 5: CONCLUSIONS

Detailed sequence analysis of the BCP was important to establish if changes occurring in this region may contribute to the high HBeAg-negativity and hepatocellular carcinoma rates observed in Black southern Africans. Serum and liver tissue from HCC patients as well as serum from ASCs were used for the analyses. Two missense mutations at positions 1809 and 1812 were found in 80% of all sequences (ASCs and HCC). These have not commonly been reported in other studies and may thus represent a wildtype sequence in southern African isolates. The commonly reported $1762^{T}1764^{A}$ missense mutations were found at a significantly higher rate in HCC patients. They were not shown to be associated with HBeAg-negativity but suppress HBeAg titers in HBeAg-positive patients, therefore suggesting that these mutations alone are not sufficient to abolish HBeAg production. Mutations at nt1753, nt1766 and nt1768 were found less commonly found. Since the BCP overlaps with the distal region of the X ORF, it is possible that different combinations of the observed mutations may change the structure and/or function of the X protein. HBX is known to be a potent transactivator of viral and cellular genes. Therefore, changes to the X protein, suppressed HBeAg expression and host immune responses, may be contributing factors in the development of hepatocarcinogenesis. Functional studies are necessary to test these hypotheses.

Chapter 6: REFERENCES

Akahane, Y., Yamanaka, T. & Suzuki, H., *et al.* 1990. Chronic active hepatitis B virus DNA and antibody against e antigen in the serum. Gastroenterology, vol. 99, pp. 1113-1119.

Ando, T. Sugiyama, K. & Goto, K., *et al.* 1999. Age at time of hepatitis Be antigen seroconversion in childhood chronic hepatitis B infection and mutant viral strain detection rates. J Pediatr Gastroenrerol Nutr, vol. 29, pp. 583-587.

Andrisani, O. M. & Barnabas, S. 1999. The transcriptional function of the hepatitis B virus X protein and its role in hepatocarcinogenesis (Review). Int J Onc, vol. 15, pp. 373-379.

Arii, M., Takada, S. & Koike, K. 1992. Identification of three essential regions of hepatitis B virus X protein for trans-activation function. Oncogene, vol. 7, pp. 397-403.

Aritomi, T., Yatsuhashi, H. & Fujino, T., *et al.* 1998. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute acute hepatitis in Japan. J Gastroenterol Hepatol, vol. 13, pp. 1125-1132.

Barlet, V., Zarski, J.P. & Thelu, M.A., *et al.* 1994. Different prevalence of precore mutants in five members of a hepatitis-B-virus-infected family: evidence for a precore variant type in an asymptomatic anti-HBs patient. J Hepatol, vol. 21, pp. 797-805.

Baumert, T.F., Rogers, S.A. & Hasegawa, K., *et al.* 1996. Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest, vol.98, pp. 2268-2276.

Baumert, T.F., Marrone, A. & Vergalla, J., *et al.* 1998. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. J Virol, vol. 72, no. 8, pp. 6785-6795.

Belshe, R.B. 1984. *Textbook of human virology*. Massachusetts: PSG publishing company, Inc. 732p.

Bläckberg, J. & Kidd-Ljunggren, K. 2000. Genotypic differences in the hepatitis B virus core promoter and precore sequences during seroconversion from HBeAg to anti-HBe. J Med Virol, vol. 60, pp.107-112.

Blum, H.E., Liang, T.J., Galun, E., *et al.* 1991. Persistence of hepatitis B viral DNA after serological recovery from hepatitis B virus infection. Hepatology, vol. 14, pp. 56-63.

Bonino, F., Brunetto, M.R. & Rizzetto, M., *et al.* 1991. Hepatitis B virus unable to secrete e antigen. Gastroenterology, vol. 100, pp.1138-1141.

Bosch,F.X. & Ribes, J. 2000. Epidemiology of liver cancer in Europe. Can J Gastroenterol, vol. 14, pp. 621-630.

Brunetto, M.R., Giarin, M.M. & Oliveri, F., *et al.* 1991. Wild-type and e antigen minus hepatitis B viruses and course of chronic hepatitis. Proc Natl Acad Sci USA, vol. 88, pp.4186-4190.

Buckwold, V.E., Xu, Z. & Chen, M., *et al.* 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol, vol.70, no.9, pp. 5845-5851.

Buckwold, V.E., Xu, Z. & Yen, B., *et al.* 1997. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. J Gen Virol, vol. 78, pp. 2055-2065.

Carman, W.F., Jacyna M.R. & Hadziyannis, S., *et al.* 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis infection. Lancet, vol. 2, 588-590.

Carman, W.F. & Thomas, H.C. 1992. Genetic variation in hepatitis B virus. Gastroenterology, vol. 102, pp. 711-719.

Carman, W., Thomas, H. & Domingo, E. 1993. Viral genetic variation: hepatitis B virus as a clinical example. Lancet, vol. 341, pp. 349-353.

Carman, W.F. 1995. Variation in the core and X genes of hepatitis B virus. Intervirology, vol. 38, pp.75-88.

Chan, H.L.Y., Hussain, M. & Lok A.S.F. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology, vol. 29, pp.976-984.

Chang, C., Ender, G. & Sprengel, R., *et al.* 1987. Expression of the precore region of an avian hepatitis B virus is not required for viral replication. J Virol, vol.61, pp. 3322-3325.

Chen, I-H., Huang, C-J. & Ting, L-P. 1995. Overlapping initiator and TATA box functions in the basal core promoter of hepatitis B virus. J Virol, vol. 69, no. 6, pp. 3647-3657.

Chen, M. & Ou, J-H. 1995. Cell type-dependent regulation of the activity of the negative regulatory element of the hepatitis B virus core promoter. Virology, vol. 214, pp. 198-206.

Cho, S.W., Shin, Y.J. & Hahm, K.B., *et al.* 1999. Analysis of the precore and core promoter DNA sequence in liver tissues from patients with hepatocellular carcinoma. J Korean Med Sci, vol. 14, pp. 424-430.

Erhardt, A., Reineke, U. & Blondin, D., *et al.* 2000. Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. Hepatology, vol. 31, pp. 716-725.

Estacio, R.C., Chavez, C.C. & Okamoto, H., *et al.* 1988. Nucleotide sequence of a hepatitis B virus genome of subtype adw isolated from a Philippino: Comparison with the reported three genomes of the same subtype. J Gastroenterol Hepatol, vol. 3, pp. 215-222.

Fang, Z-L., Ling, R. & Wang, S.S., *et al.* 1998. HBV core promoter mutations prevail in patients with hepatocellular carcinoma from Guangxi, China. J Med Virol, vol.56, pp. 18-24.

Feitelson, M.A. 1994. Biology of disease. Biology of hepatitis B virus variants. Lab Invest, vol. 71, no. 3, pp. 324-349.

Fields, B.N., Knipe, D.M. & Chanock, R.M., *et al.* 1985. *Virology*. New York: Raven Press. 1384p.

Freidt, M., Gerner, P. & Lausch, E., *et al.* 1999. Mutations in the basic core promoter and the precore region of hepatitis B virus and their selection in children with fulminant and chronic hepatitis B. Hepatology, vol. 29, pp. 1252-1258.

Fukuda, R., Xuan-Thanh, N. & Ishimura, N., *et al.* 1995. X gene and precore region mutations in the hepatitis B virus genome in persons positive for antibody to hepatitis B e antigen: Comparison between asymptomatic "healthy" carriers and patients with severe chronic active hepatitis. J Infect Dis, vol. 172, pp. 1191-1197.

Fukuda, R., Ishimura, N. & Kushiyama, Y., *et al.* 1996. Hepatitis B virus with X gene mutation is associated with the majority of serologically "silent" non-B, non-C chronic hepatitis. Microbiol Immunol, vol. 40, no. 7, pp. 481-488.

Galibert, F., Mandart, E. & Fitoussi, F., et al. 1979. Nucleotide sequence of the hepatitis B virus genome (subtype *ayw*) cloned in *E. coli*. Nature, vol.281, pp. 646-650.

Ganem, D. & Varmus, H.E. 1987. The molecular biology of the hepatitis B viruses. Ann Rev Biochem, vol. 56, pp. 51-693.

Gerber, M.A. & Thung, S.N. 1985. Biology of disease. Molecular and cellular pathology of hepatitis B. Lab Invest, vol. 52, no. 6, pp. 572-590.

Gerlach, K.K. & Schloemer, R.H. 1992. Hepatitis B virus C gene promoter is under negative regulation. Virology, vol. 189, pp. 59-66.

Gotoh, K., Mima, S. & Uchida, T., *et al.* 1995. Nucleotide sequence of hepatitis B virus isolated from subjects without serum anti-hepatitis B core antibody. J Med Virol, vol. 46, pp. 201-206.

Guidotti, L.G., Matzke, B. & Pasquinelli, C., *et al.* 1996. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. J Virol, vol. 70, no. 10, pp. 7056-7061.

Günther, S., Piwon, N. & Iwanska, A. *et al.* 1996. Type, prevalence, and significance of core promoter/ enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. J Virology, vol. 70, no.12, pp. 8318-8331.

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

Guo, W., Chen, M. & Yen, T.S.B., *et al.* 1993. Hepatocyte-specific expression of the hepatitis B virus core promoter depends on both positive and negative regulation. Mol Cell Biol, vol 13, no. 1, pp. 443-448.

Hasegawa, K., Huang, J. & Rogers, S.A., *et al.* 1994. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J Virol, vol. 68, no. 3, pp. 1651-1659.

Hiraga, M., Nishizono, A. & Mifune, K., *et al.* 1994. Analysis of upsteam region of hepatitis B virus core gene using in vitro transcription system. J Med Virol, vol.43, pp.404-411.

Honda, A., Yokosaka, O. & Ehata, T., *et al.* 1999. Detection of mutations in the enhancer 2/core promoter region of hepatitis B virus in patients with chronic hepatitis B virus infection: Comparison with mutations in precore and core regions in relation to clinical status. J Med Virol, vol. 57, pp. 337-344.

Honigwachs, J., Faktor, O. & Dikstein, R., *et al.* 1989. Liver-specific expression of hepatitis B virus is determined by the combined action of the core gene promoter and the enhancer. J Virol, vol. 63, pp 919-924.

Horikita, M., Itoh, S. & Yamamoto, K., *et al.* 1994. Differences in the entire nucleotide sequence between hepatitis B virus genomes from carriers positive for antibody to hepatitis B e antigen with and without active disease. J Med Virol, vol. 44, pp. 96-103.

Hou, J. Lau, G.K.K. & Cheng J., *et al.* 1999. T_{1762}/A_{1764} variants of the basal core promoter of hepatitis B virus; serological and clinical correlations in Chinese patients. Liver, vol. 19, pp. 411-417.

Howard, C.R. 1995 The structure of hepatitis B envelope and molecular variants of hepatitis B virus. J Viral Hepatitis, vol. 2, pp. 165-170.

Hsia, C.C., Yuwen, H. & Tabor, E. 1996. Hot-spot mutations in hepatitis B virus X gene in hepatocellular carcinoma. Lancet, vol. 348, pp. 625-626.

Hsia, C.C., Nakashima, Y. & Tabor, E. 1997. Deletion mutants of the hepatitis B virus X gene in human hepatocellular carcinoma. Biochem Biophys Res Commun, vol. 241, pp. 726-729.

IARC monographs on the evaluation of carcinogenic risks to humans, volume 59, Hepatitis viruses. 1994. Lyon, France. pp. 54-57.

Inoue, K., Yoshiba, M. & Sekiyama, K., *et al.* 1998. Clinical and molecular virological differences between fulminant hepatic failures following acute and chronic infection with HBV. J Med Virol, vol. 55, pp. 35-41.

Johnson, J.L., Raney, A.K. & McLachlan, A. 1995. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. Virology, vol. 208, pp. 147-158.

Junker-Niepmann, M., Bartenschlager, R. & Schaller, H. 1990. A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J, vol. 9, pp. 3389-3396.

Kanai, K., Kako, M. & Aikawa, T., *et al.* 1996. Core promoter mutations of hepatitis B virus for the response to interferon in e antigen-positive chronic hepatitis B. Am J Gastroenterol, vol. 91, no. 10, pp. 2150-2156.

Kaneko, M., Uchida, T. & Moriyama, M., *et al.* 1995. Probable implication of mutations in the X open reading frame in the onset of fulminant hepatitis B. J Med Virol, vol. 47, pp. 204-208.

Kew, M.C., Marcus, R.G. & Macnab G.M., *et al.* 1975. Hepatitis B (surface) antigen in mothers and their infants. SAMJ, vol.49, no. 23, pp. 1471-1473.

Kew, M.C., Mackay, M.E. & Mindel, B., *et al.* 1976. Prevalence of hepatitis-B surface antigen and antibody in white and black patients with diabetes mellitus. J Clin Microbiol, vol. 4, pp. 467

Kew, M.C. 1994. Epidemiology of hepatocellular carcinoma. Viral hepatitis and liver disease. pp. 681-684.

Kew, M.C. 1996. Hepatitis B and C viruses and hepatocellular carcinoma. Clin Lab Med, vol. 16, no. 2, pp. 395-406.

Kidd, A.H. & Kidd-Ljunggren, K. 1996. A revised secondary structure model for the 3'-end of hepatitis B virus pregenomic RNA. Nucleic Acids Res, vol. 24, no. 17, pp. 3295-3301.

Kidd-Ljunggren, K., Öberg, M. & Kidd, A.H. 1995. The hepatitis B virus X gene: analysis of functional domain variation and gene phylogeny using multiple sequences. J Gen Virol, vol. 76, pp. 2119-2130.

Kidd-Ljunggren, K., Öberg, M. & Kidd, A.H. 1997. Hepatitis B virus X gene 1751 to 1764 mutations: implications for HBeAg status and disease. J Gen Virol, vol. 78, pp. 1469-1478.

Kim, Y-H., Kanh, S-K. & Lee, Y.I. 1993. Functional analysis of hepatitis B virus trabsactivator X: Implication of the leucine zipper-like region and C-terminal seven conserved amino acids in functional regions. Biochem Biophys Res Commun, vol. 197, no.2, pp. 894-903.

Kim, Y.S., Kim S. & Hwang S.G. *et al.* 1999. Diversity of core promoter mutations in immune clearance phase of chronic HBV infection. Eur J Gastroenterol Hepatol, vol. 11, pp. 821-825.

Koike, K. & Takada, S. 1995. Biochemistry and functions of hepatitis B virus X protein. Intervirology, vol. 38, pp.89-99.

Kramvis, A., Bukofzer, S. & Kew, M.C., *et al.* 1997. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. Hepatology, vol. 25, pp. 235-240.

Kramvis, A., Kew, M.C., & Bukofker, S. 1998a. Hepatitis B virus precore mutants in serum and liver of Southern African blacks with hepaticellular carcinoma. J Hepatol, vol 28, pp. 132-141.

Kramvis, A. & Kew, M.C. 1998b. Structure and function of the encapsidation signal of hepadnaviridae. J Viral Hepatitis, vol. 5, pp. 357-367.

Kramvis, A. & Kew, M.C. 1999. The core promoter of hepatitis B virus. J of Viral hepatitis, vol. 6, pp. 415-427.

Kurosaki, M., Enomoto, N. & Asahina, Y., *et al.* 1996. Mutations in the core promoter region of the hepatitis B virus in patients with chronic hepatitis B. J Med Virol, vol. 49, pp.115-123.

Kwok, S. & Higuchi, R. 1989. Avoiding false positives with PCR. Nature, vol. 339, pp. 237-238.

Lamberts, C., Nassal, M. & Velhagen, I., *et al.* 1993. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J Virol, vol. 67, no.7, pp. 3756-3762.

Laskus, T., Rakela, J. & Tong, M.J., *et al.* 1994a. Naturally occurring hepatitis B virus mutants with deletions in the core promoter region. J Hepatol, vol. 20, pp. 837-841.

Laskus, T., Rakela, J. & Steers, J.L., *et al.* 1994b. Precore and contiguous regions of hepatitis B virus in liver transplantation for end-stage hepatitis B. Gastroenterology, vol. 107, pp. 1774-1780.

Laskus, T., Rakela, J. & Nowicki, M.J., *et al.* 1995. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology, vol. 109, pp. 1618-1623.

Laskus, T., Wang, L.F. & Radkowski, M., *et al.* 1997. Comparison of hepatitis B virus core promoter sequences in peripheral blood mononuclear cells and serum from patients with hepatitis B. J Gen Virol, vol. 78, pp. 649-653.

Li, J., Buckwold, V.E. & Hon, M-W., *et al.* 1999. Mechanism of suppression of HBV precore RNA transcription by a frequent double mutation. J Virol, vol. 73, no. 2, pp 1239-1244.

Li, M., Xie,Y. & Wu X., *et al.* 1995. HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. Virology, vol. 214, pp. 371-378.

Lindh, M., Gustavson, C. & Mardberg, K., *et al.* 1998. Mutation of nucleotide 1,762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. J Med Virol, vol.55, pp.185-190.

Lindh, M., Hannoun, C. & Dhillon A.P., *et al.* 1999. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J Infect Dis, vol. 179, pp 775-782.

López-Cabrera, M., Letovsky, J. & Hu, K-Q., *et al.* 1990. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: Trans-activation and repression by CCAAT/enhancer binding protein. Proc Natl Acad Sci USA, vol. 87, pp. 5069-5073.

Lo, W-Y. & Ting, L-P. 1994. Repression of enhancer II activity by a negative regulatory element in the hepatitis B virus genome. J Virol, vol. 68, no.3, pp. 1758-1764.

Loncarevic, I.F., Schranz, P. & Zentgraf, H., *et al.* 1990. Replication of hepatitis B virus in a hepatocellular carcinoma. Virology , vol. 174, pp. 158-168.

Mayerat, C., Mantegani, A. & Spertini, F., *et al.* 1999. Mutations in the basal core promoter and precore/core gene of hepatitis B virus in patients with chronic active but not acute hepatitis B. Eur J Clin Microbiol Infect Dis, vol. 18, pp. 871-878.

McMillian, J.S., Bowden, D.S. & Angus, P.W., *et al.* 1996. Mutations in the hepatitis B virus precore/core gene and core promoter in patients with severe recurrent disease following liver transplantation. Hepatology, vol. 24, pp. 1371-1378.

Milich, D.R., Jones, J.E. & Hughes, J.L., *et al.* 1990. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proc Natl Acad Sci USA, vol. 87, pp. 6599-6603.

Miller, S.A., Dykes, D.D. & Polesky, H.F. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res, vol. 16, no. 3, pp. 1215.

Miyakawa, Y., Okamoto, H. & Mayumi, M. *et al.* 1997. The molecular basis of hepatitis B e antigen (HBeAg)-negative infections. J Viral hepatitis, vol. 4, no. 4, pp. 1-8.

Moriyama, K., Takada, T. & Tsutsumi, Y., *et al.* 1994. Mutations in the transcriptional regulatory region of the precore and core/pregenome of a hepatitis B virus with defective HBeAg production. Fukuoka Acta Medica, vol. 85, no. 11, pp. 314-322.

Moriyama, K., Okamoto, H. & Tsuda, F., *et al.* 1996. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology, vol. 226, pp. 269-280.

Moriyama, K. 1997. Reduced antigen production by hepatitis B virus harbouring nucleotide deletions in the overlapping X gene and precore-core promoter. J Gen Virol, vol. 78, pp. 1479-1489.

Nagasaka, A., Hige, S., & Marutani, M. *et al.* 1998. Prevalence of mutations in core promoter/precore region in Japanese patients with chronic hepatitis B virus infection. Dig Dis Sci, vol. 43, no. 11, pp. 2473-2478.

Nishizono, A., Hiraga, M. & Kohno, K., *et al.* 1995. Mutations in the core promoter/enhancer II regions of naturally occurring hepatitis B virus variants and analysis of the effects on transcription activities. Intervirology, vol. 38, pp. 290-294.

Ogata, N., Miller, R.H. & Ishak, K.G., *et al.* 1993. The complete nucleotide sequence of a pre-core mutant hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology, vol. 194, pp. 263-276.

Okamoto, H., Yotsumoto, S. & Akahane, Y., *et al.* 1990. Hepatitis B viruses with precore region detects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol, vol. 64, pp. 1298-1303.

Okamoto, H., Tsuda, F. & Akahane, Y., *et al.* 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol, vol. 68, no. 12, pp. 8102-8110.

Orito, E., Mizokami, M. & Ina, Y., *et al.* 1989. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. Proc Natl Acad Sci USA, vol. 86, pp. 7059-7062.

Park, Y-M., Kim, B-S. & Tabor, E. 1997. Precore codon 28 stop mutation in hepatitis B virus patients with hepatocellular carcinima. Kor J Intern Med, vol.12, no. 2, pp. 201-207.

Preisler-Adams, S., Schlayer, H-J. & Peters, T., *et al.* 1993. Sequence analysis of hepatitis B virus DNA in immunologically negative infection. Archi Virol, vol. 133, pp. 385-396.

Pult, I., Chouard, T. & Wieland, S., *et al.* 1997. A hepatitis B virus mutant with a new hepatocyte nuclear factor 1 binding site emerging in transplat-transmitted fulminant hepatitis B. Hepatology, vol. 25, pp. 1507-1515.

Radzwill, G., Tucker, W. & Schaller, H. 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H actiuvity. J Virol, vol. 64, pp. 613-620.

Raney, A.K., Jonhson, J.L. & Palmer, C.N.A., *et al.* 1997. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J Virol, vol. 71, no. 2, pp. 1058-1071.

Repp, R., Keller, C. & Borkhardt, A., *et al.* 1992. Detection of a hepatitis B virus variant with a truncated X gene and enhancer II. Arch Virol, vol. 125, pp. 299-304.

Ritter, S.E., Whitten, T.M. & Quets, A.T., *et al.* 1991. An internal domain of the hepatitis B virus X antigen is necessary for transactivating activity. Virology, vol. 182, pp. 841-845.

Robert, G. "Robert's HBV page." Epidemiology of hepatitis B. 1998. <http:\\www.globalserve.net/~harlequin/HBV/epiden.html> (2 June 1998).

Runkel, L., Fischer, M. & Schaller, H. 1993. Two-codon insertion mutations of the HBX define two separate regions necessary for its trans-activation function. Virology, vol. 197, pp.529-536.

Sato, S., Suzuki, K. & Akahane, Y., *et al.* 1995. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med, vol. 122, no. 4, pp. 241-248.

Scaglioni, P.P., Melegari, M. & Wands, J.R. 1997a. Posttranscriptional regulation of hepatitis B virus replication by the precore protein. J Virol, vol. 71, no. 1, pp. 345-353.

Scaglioni, P.P., Melegari, M. & Wands, J.R. 1997b. Biological properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. Virology, vol. 233, pp. 374-381.

Schlicht, H.J., Salfeld, J. & Schaller, H. 1987. The duck hepatitis B virus pre-C region encodes a signal sequence which is essential for synthesis and secretion of processed core particles but not for viral formation. J Virol, vol. 61, pp. 2208-2212.

Schaller, H. & Fischer, M. 1991. Transcriptional control of hepadnavirus gene expression. Cur Topics Micro Immun, vol. 168, pp. 21-39

Schoub, B.D. 1994. A primer of hepatitis serology. Virus SA, vol. 3, no. 1, pp. 1-7.

Shindo, M. Hamada, K. & Koya, S., et al. 1999. The clinical significance of core promoter and precore mutations during the natural course and interferon therapy in patients with chronic hepatitis B. Am J Gastroenterol, vol. 94, no. 8, pp. 2237-2245).

Sterneck, M., Günther, S. & Santantonio, T., *et al.* 1996. Hepatitis B virus genomes of patients with fulminant hepatiis do not share a specific mutation. Hepatology, vol. 24, no. 2, pp. 300-306.

Takada, S. & Koike, K. 1994. Three sites of the hepatitis B virus X protein cooperatively interact with cellular proteins. Virology, vol. 205, pp. 503-510.

Takahashi, K., Aoyama, K. & Ohno, N., *et al.* 1995. The precore/core promoter mutant ($T^{1762}A^{1764}$) of hepatitis B virus: clinical significance and an easy method for detection. J Gen Virol, vol. 76, pp. 3159-3164.

Takahashi, K., Akahane, Y. & Hino, K., *et al.* 1998. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. Arch Virol, vol. 143, pp. 2313-2326.

Takahashi, K., Ohta, Y. & Kanai, K., *et al.* 1999. Clinical implications of mutations C-to-T^{1653} and T-to-C/A/G^{1753} of hepatitis B virus genotype C genome in chronic liver disease. Arch Virol, vol. 144, pp. 1299-1308.

Thomas, H.C. & Carman, W.F. 1994. Envelope and precore/core variants of hepatitis B virus. Gastroenterology Clinics of North America, vol. 23, no. 3, pp. 499-514.

Tiollais, P., Pourcel, C. & Dejean, A. 1985. The hepatitis B virus. Nature, vol. 317, pp. 489-496.

Tiollais, P., Buendia, M-A. & Bréchot, C., *et al.* 1988. Structure, genetic organization, and transcription of Hepadna viruses. Viral Hepatitis and Liver Disease, pp. 295-300.

Tong, S., Li, J. & Vitvitski, L., *et al.* 1990. Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. Virology, vol. 176, pp. 596-603.

Uchida, T., Shimojima, M. & Gotoh, K., *et al.* 1994a. "Silent" hepatitis B virus mutants are responsible for non-A, non-B, non-C, non-D, non-E hepatitis. Microbiol Immunol, vol. 38, no. 4, pp. 281-285.

Uchida, T., Aye, T.T. & Shikata, T., *et al.* 1994b. Evolution of the hepatitis B virus gene during chronic infection in seven patients. J Med Virol, vol. 43, pp. 148-154.

Uchida, T., Gotoh, K. & Shikata, T. 1995. Complete nucleotide sequences and the characteristics of two hepatitis B virus mutants causing serologically negative acute or chronic hepatits B. J Med Virol, vol. 45, pp. 247-252.

Uchida, T., Saitoh, T. & Shinzawa, H. 1997. Mutations of the X region of hepatitis B virus and their clinical implications. Pathology International, vol. 47, pp. 183-193.

Vyas, G.N., Dienstag, J.L. & Hoofnagle, J.H. 1984. *Viral Hepatitis and liver disease.* New York, London: Grune and Stratton Inc. Harcourt Brace Jovanovich, Publishers.

Wang,Y., Chen, P. & Wu, X., *et al.* 1990. A new enhancer element, ENII, identified in the X gene of hepatitis B virus. J Virol, vol. 64, no. 8, pp. 3977-3981.

Will, H., Reiser, W. & Weimer, T., *et al.* 1987. Replication strategy of human hepatitis B virus. J Virol, vol. 61, no.3, pp. 904-911.

Wu, X., Zhu, L. & Li, Z-P., *et al.* 1992. Functional organization of enhancer (ENII) of hepatitis B virus. Virology, vol. 191, pp. 490-494.

Yaginuma, K., Shirakata, Y. & Kobayashi, M., *et al.* 1987. Hepatitis B virus (HBV) particles are produced in a cell culture system by transsient expression of transfected HBV DNA. Proc Natl Acad Sci USA, vol. 84, pp. 2678-2682.

Yaginuma, K. & Koike K. 1989. identification if a promoter region for 3.6-kilobase mRNA of hepatitis B virus and specific cellular binding protein. J Virol, vol. 63, no. 7, pp. 2914-2920.

Yee, C.T. 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science, vol. 24, pp. 658-661.

Yen, T.S.B. 1993. Regulation of hepatitis B virus gene expression. Seminars in Virology, vol. 4, pp. 33-42.

Yu, X. & Mertz, J.E. 1996. Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. J Virol, vol. 70, no. 12, pp. 8719-8726.

Yu, X. & Mertz, J.E. 1997. Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. J Virol, vol. 71, no. 12, pp. 9366-9374.

Yuh, C-H. & Ting, L-P. 1990. The genome of hepatitis B virus contains a second enhancer: Cooperation of two elements within this enhancer is required for its function. J Virol, vol. 64, no. 9, pp. 4281-4287.

Yuh, C-H. & Ting, L-P. 1991. C/EBP-like proteins binding to the functional box- a nd box- of the second enhancer of hepatitis B virus. Mol Cell Biol, vol. 11, no. 10, pp. 5044-5052.

Yuh, C.H., Chang, Y.L. & Ting, L.P. 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J Virol, vol. 66, no. 7, pp. 4073-4084.

Yuh, C-H. & Ting, L-P. 1993. Differentiated liver cell specificity of the second enhancer of hepatitis B virus. J Virol, vol. 67, no. 1, pp. 142-149.

Zhang, P., Raney, A. K. & McLachlan, A. 1993. Characterization of functional Sp1 transcription factor binding sites in hepatitis B virus nucleocapsid promoter. J Virol, vol. 67, pp. 1472-1481.

Zhang, P. & McLachlan, A. 1994. Differentiation-specific transcriptional regulation of the hepatitis B virus nucleocapsid gene in human hepatoma cell lines. Virology, vol. 202, pp. 430-440.

Zhou, D-X. & Yen, T.S.B. 1990. Differential regulation of the hepatitis B virus surface gene promoters by a second viral enhancer. J Biol Chem, vol. 265, no. 34, pp. 20731-20734.

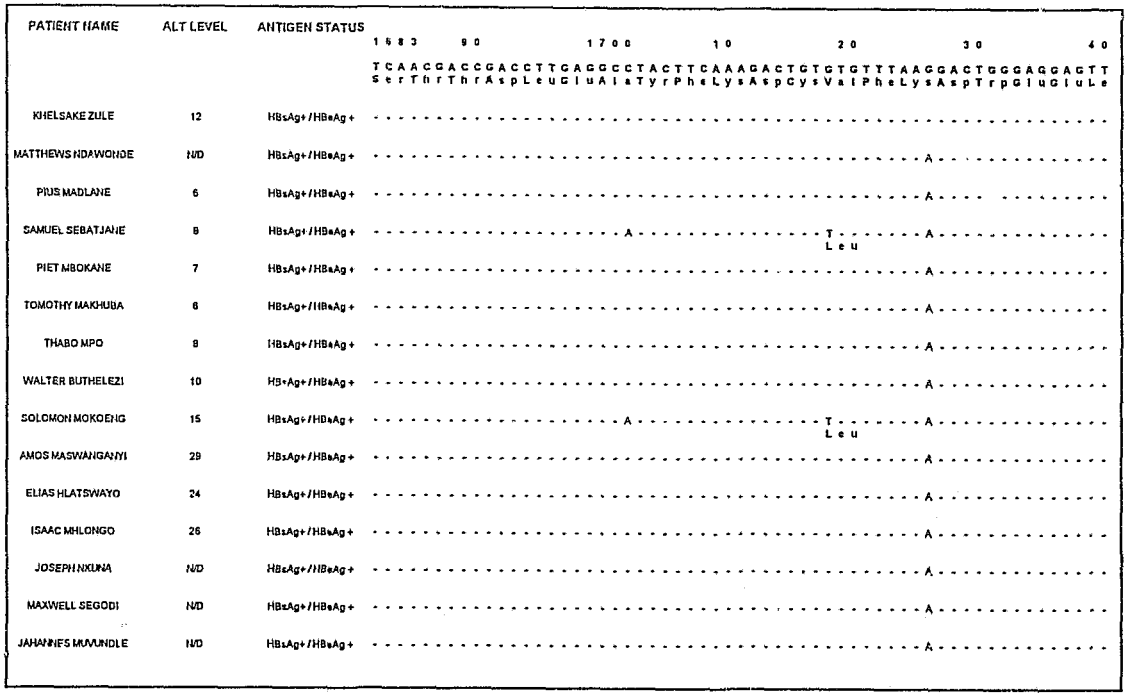


Figure 15 Nucleotide and deduced amino acid sequence of the enhancer II (1683-1742) of HBV DNA isolated from 15 HBeAg positive sera of ASCs aligned to sequence EMBL X70185 (shown in full). Where available, ALT levels and/or patients' HBV antigen status are given.

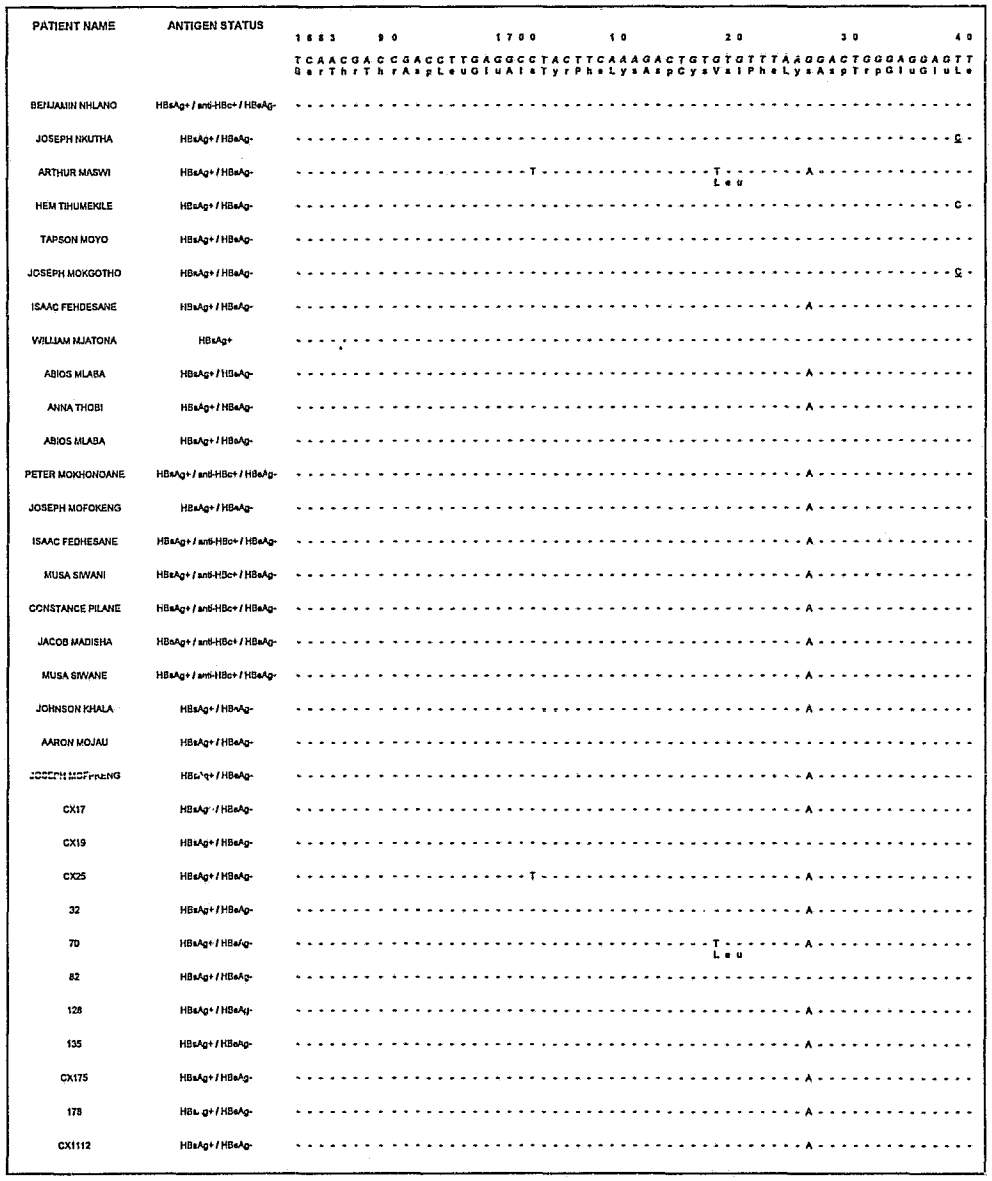


Figure 16 Nucleotide and deduced amino acid sequence of the enhancer II (1683-1742) of HBV DNA isolated from 32 HBeAg-negative sera of ASCs aligned to sequence EMBL X70185 (shown in full). Patients' HBV antigen status is given. Substitutions that occurred together with wildtype nucleotides are underlined.

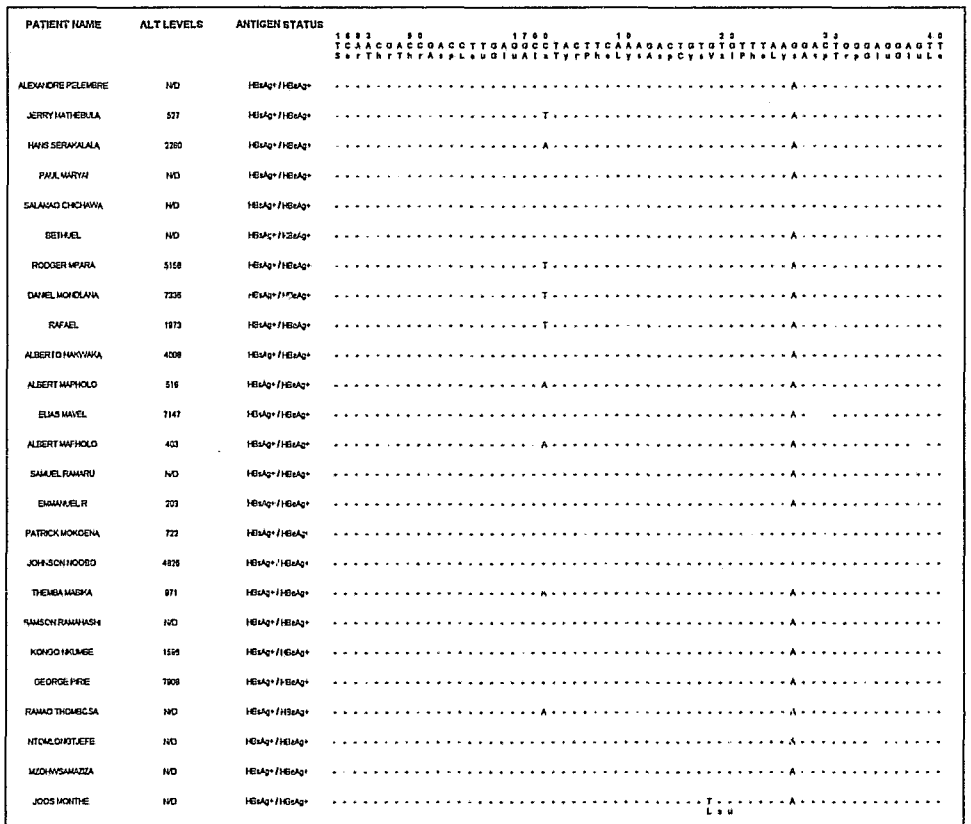


Figure 17 Nucleotide and deduced amino acid sequence of the enhancer II (1683-1742) of HBV DNA isolated from 25 HBeAg-positive sera of HCC patients aligned to sequence EMBL X70185 (shown in full). Where available, ALT levels and/or patients' HBV antigen status are given.

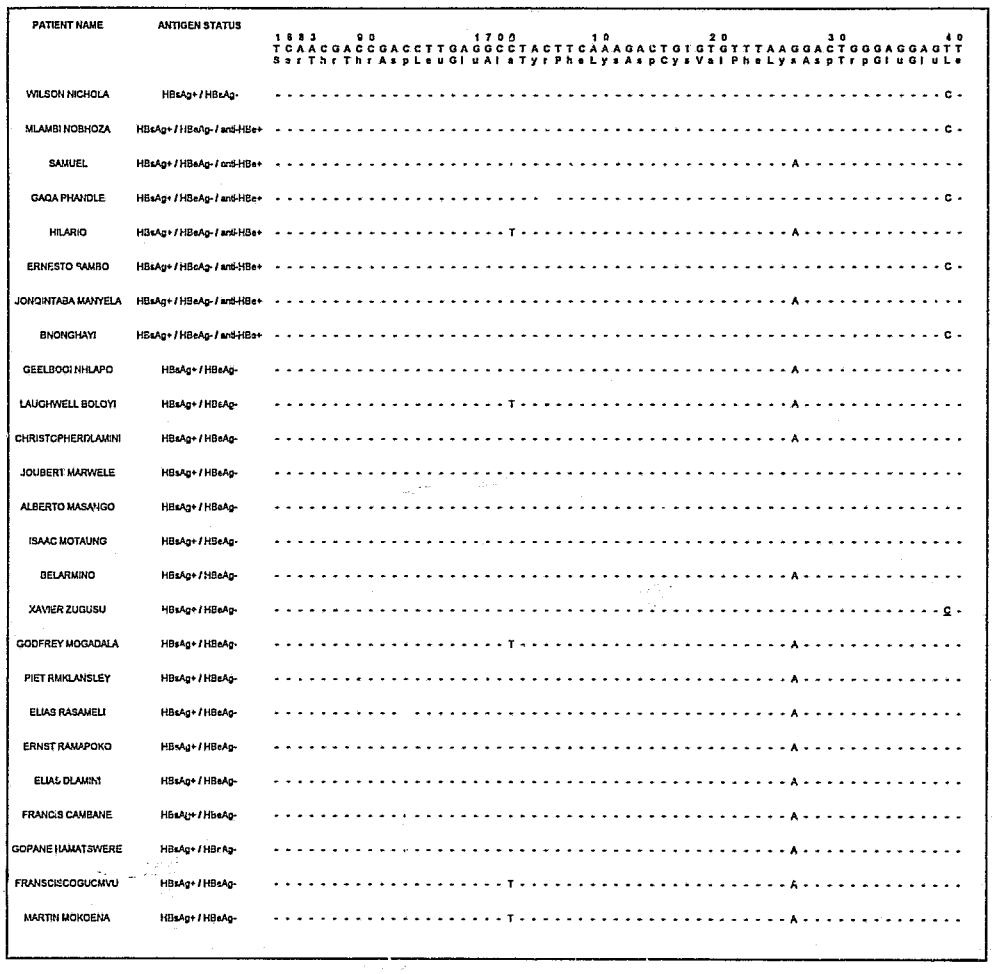


Figure 18 Nucleotide and deduced amino acid sequence of the enhancer II (1683-1742) of HBV DNA isolated from 25 HBeAg-negative sera of HCC patients aligned to sequence EMBL X70185 (shown in full). Patients' HBV antigen status is given. Substitutions that occurred together with wildtype nucleotides are underlined.

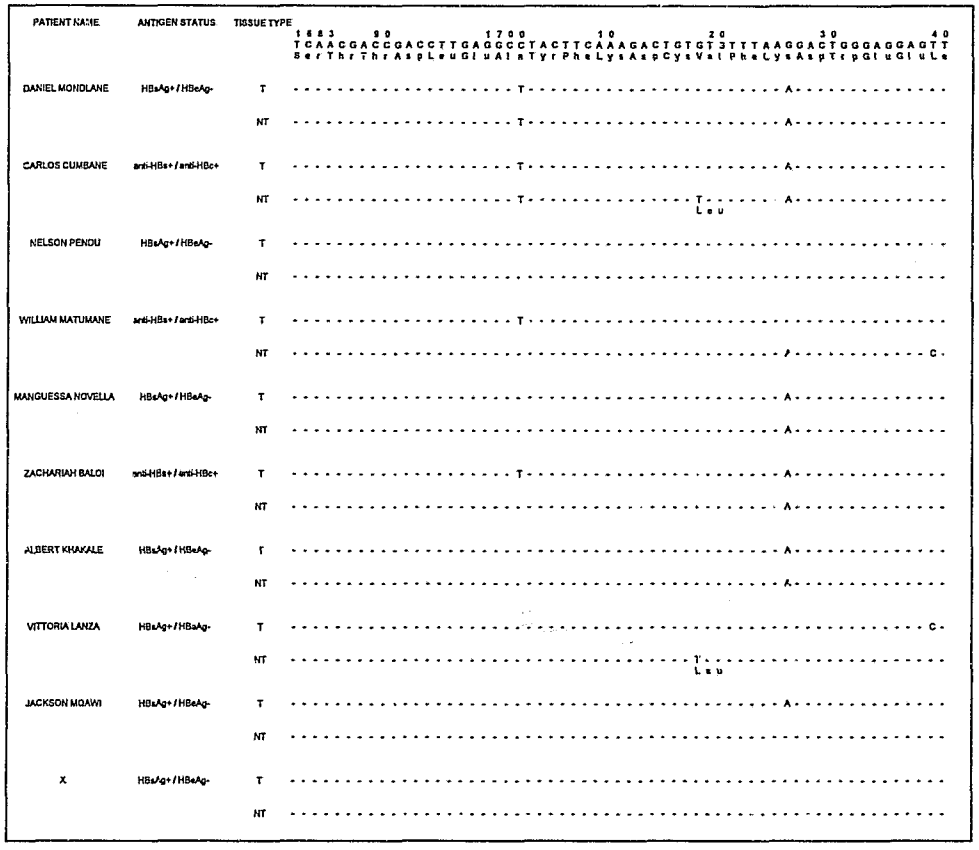


Figure 19 Nucleotide and deduced amino acid sequence of the enhancer II (1683-1742) of HBV DNA isolated from 10 nontumorous and corresponding tumorous liver tissues aligned to sequence EMBL X70185 (shown in full). Patients' HBV antigen status are given.

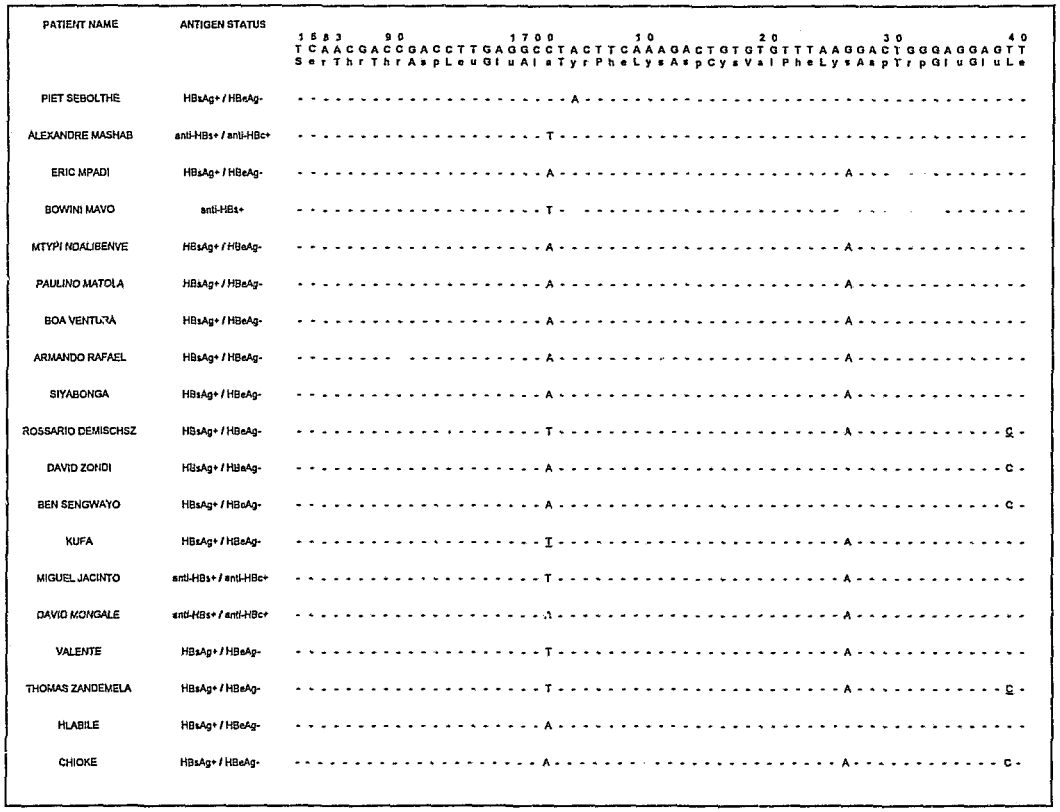


Figure 20 Nucleotide and deduced amino acid sequence of the enhancer II (1683-1742) of HBV DNA isolated from 19 tumorous liver tissues aligned to sequence EMBL X70185 (shown in full). Patients' HBV antigen status are given. *Substitutions that occurred together with wildtype nucleotides are underlined.*

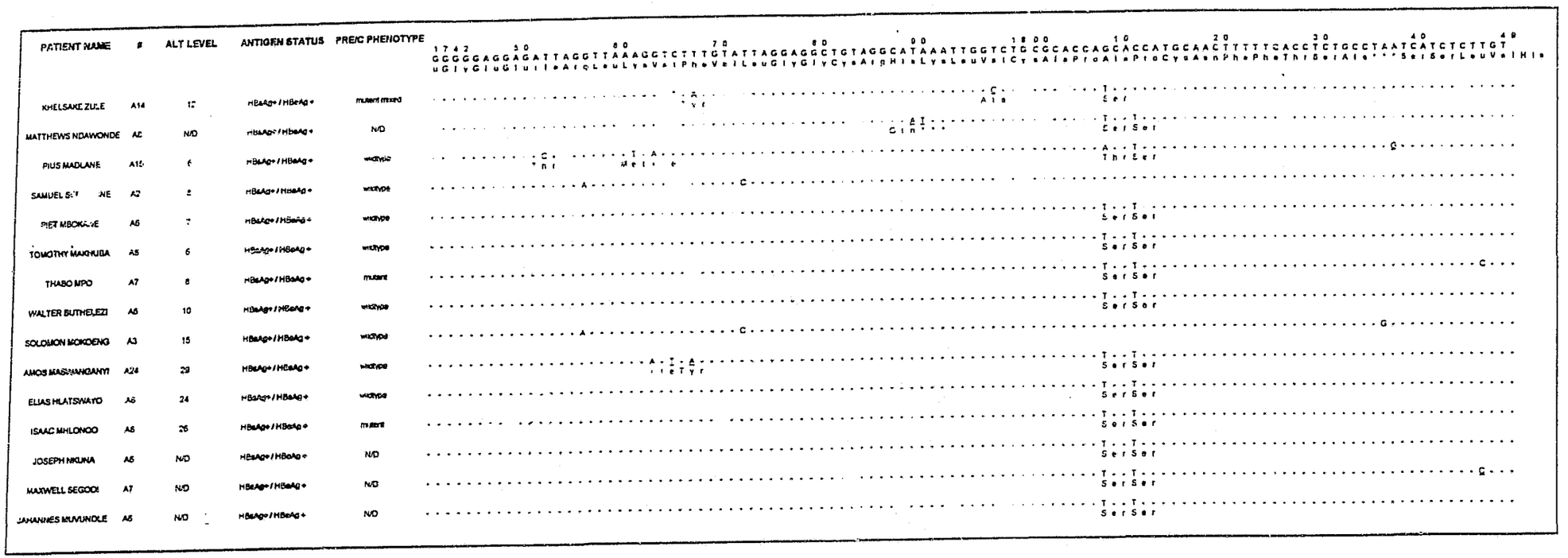


Figure 21 Nucleotide and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from 15 HBeAg-positive sera of ASCs aligned to sequence EMBL X7018 (shown in full). Sequence number, ALT levels, patients' HBV antigen status and/or phenotype of precore region are given. Precore region phenotype results from previous study by Kramvis, *et al.*, 1997, 1998. Substitutions that occurred together with wildtype nucleotides are underlined.

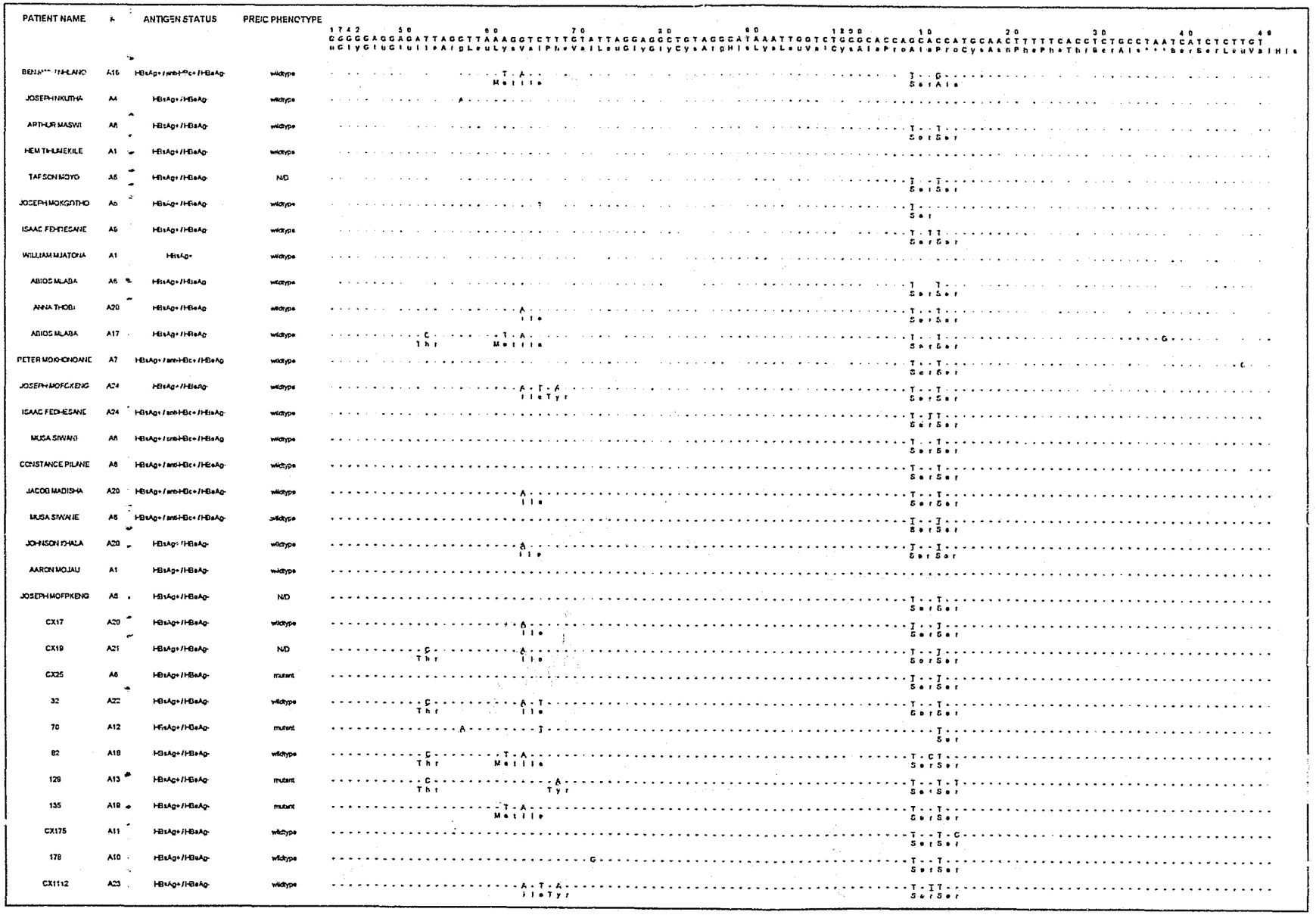


Figure 22 Nucleotide and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from 32 HBeAg-negative sera of ASCs aligned to sequence EMBL X7018 (shown in full). Sequence number, patients' HBV antigen status and/or phenotype of precore region are given. Precore region phenotype results from previous study by Kramvis, *et al.*, 1997, 1998. Substitutions that occurred together with wildtype nucleotides are underlined.

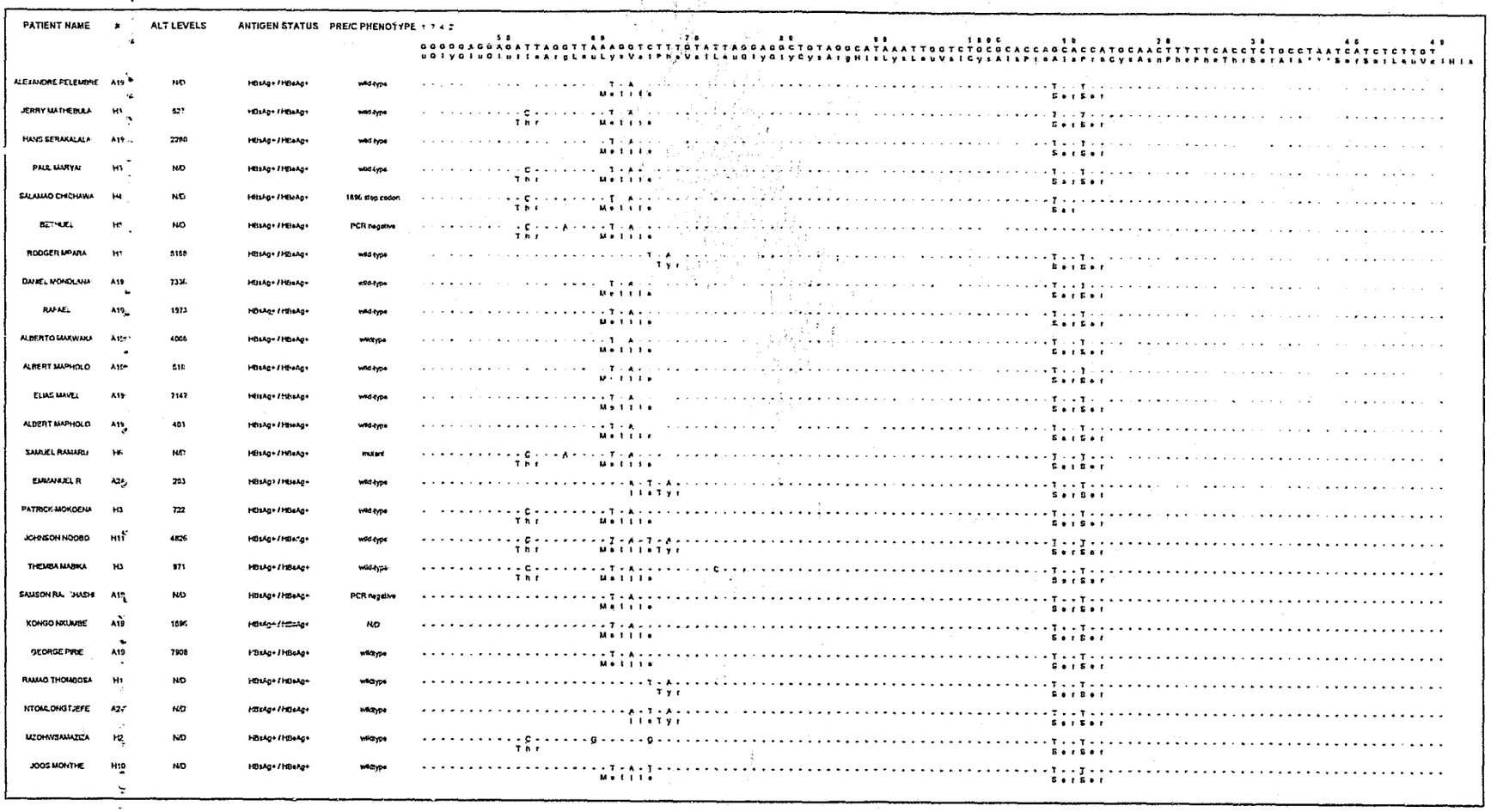


Figure 23 Nucleotide and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from 25 HBeAg-positive sera of HCC patients aligned to sequence EMBL X7018 (shown in full). Sequence number, ALT levels, patients' HBV antigen status and/or phenotype of precore region are given. Precore region phenotype results from previous study by Kramvis, *et al*., 1997, 1998. Substitutions that occurred together with wildtype nucleotides are underlined.

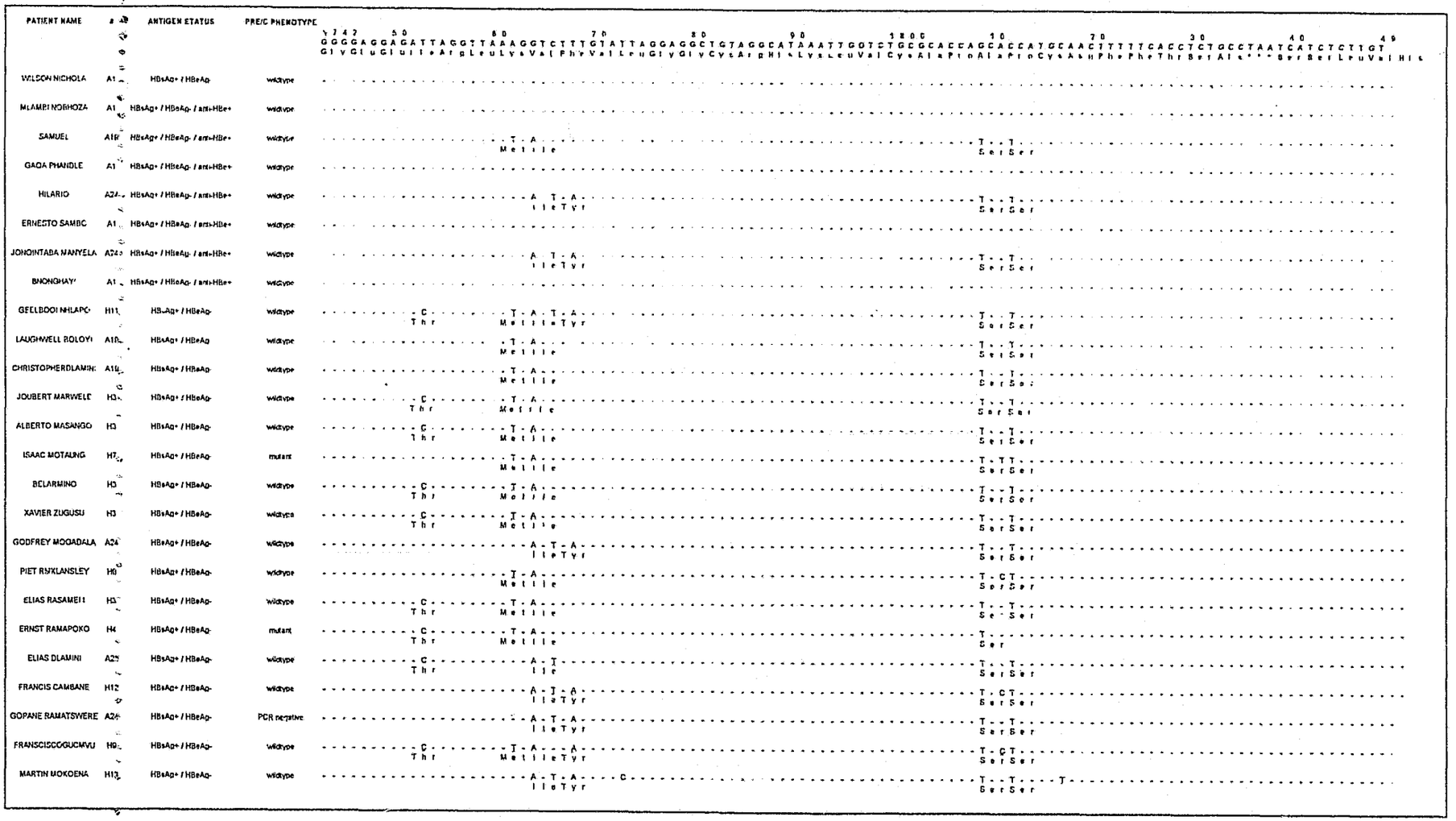


Figure 24 Nucleotide and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from 25 HBeAg-negative sera of HCC patients aligned to sequence EMBL X7018 (shown in full). Sequence number, patients' HBV antigen status and/or phenotype of precore region are given. Precore region phenotype results from previous study by Kramvis, *et al*., 1997, 1998. Substitutions that occurred together with wildtype nucleotides are underlined.

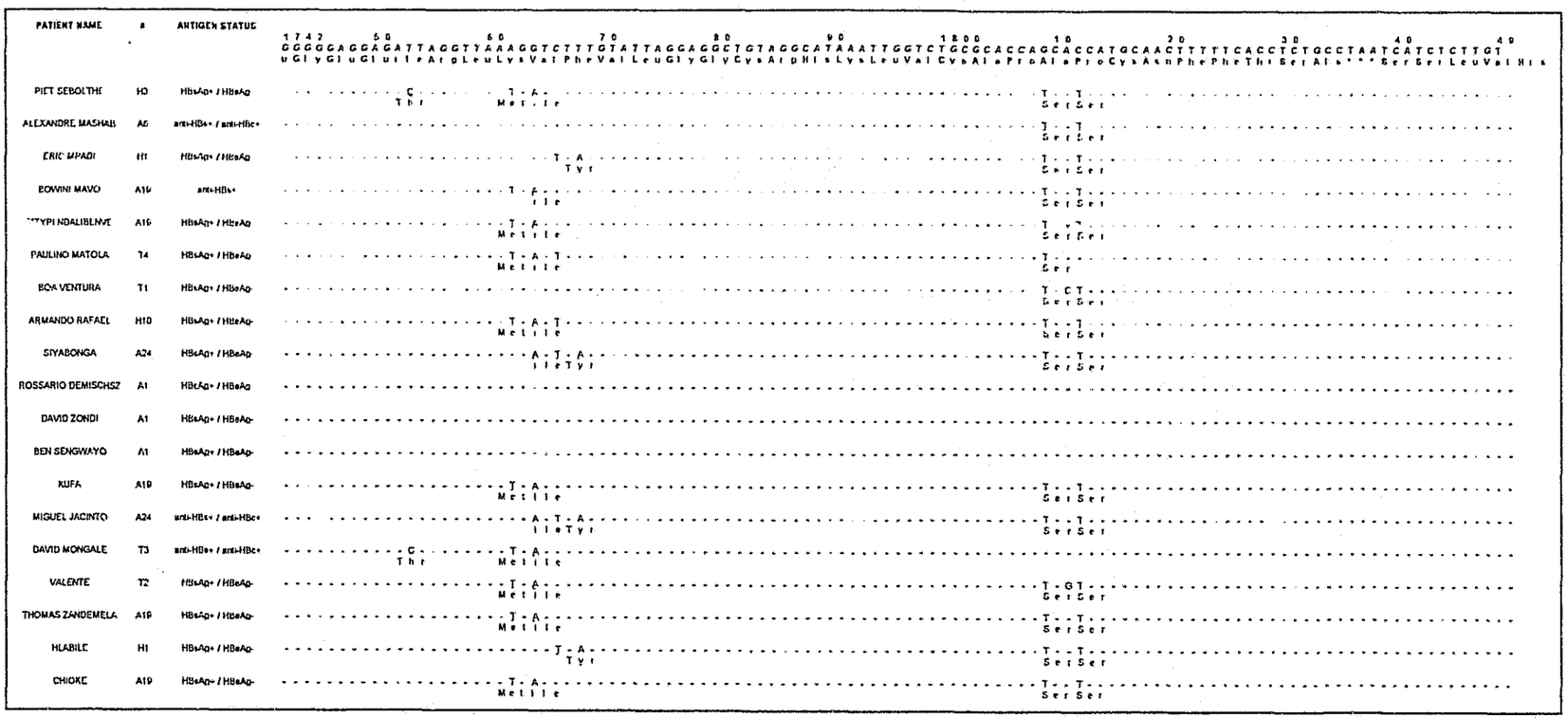


Figure 25 Nucleotide and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from 19 tumorous liver tissues aligned to sequence EMBL X7018 (shown in full). Sequence number and patients' HBV antigen status are given. Substitutions that occurred together with wildtype nucleotides are underlined.

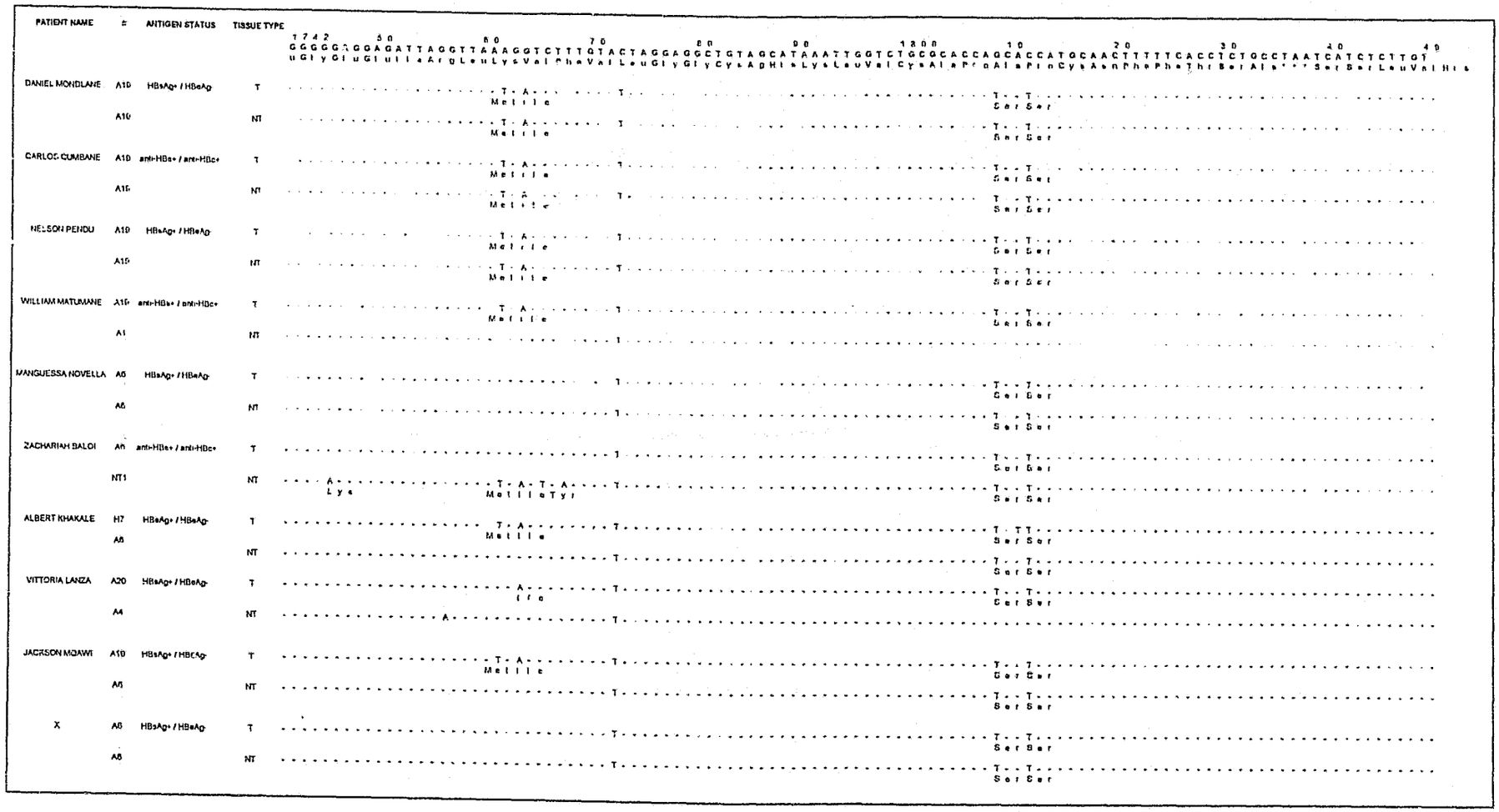


Figure 26 Nucleotide and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from 10 nontumorous and their corresponding tumorous liver tissues aligned to sequence EMBL X7018 (shown in full). Sequence number and patients' HBV antigen status are given. Substitutions that occurred together with wildtype nucleotides are underlined.

Appendix B: Solutions

2% AGAROSE GEL

- 2g molecular grade agarose (Whitehead Scientific)
- 100ml 1x TBE running buffer
- Boil in microwave until all the agarose is dissolved
- Cool the agarose to about 55°C
- Add 3.5µL of ethidium bromide and mix thoroughly
- Pour into gel tray and allow to set for at least 30 minutes

0.1% AMMONIUM PERSULPHATE

- 0.1g ammonium persulphate (BIO-RAD)
- 1mL best quality water
- Dissolve
- If storing, keep in the dark at -20°C

40% BIS-ACRYLAMIDE STOCK

- 38g acrylamide (Promega)
- 2g bis-acrylamide (Promega)
- ~50ml best quality water
- Stir at room temperature, until completely dissolved
- While stirring keep in the dark (by covering container with foil)
- Make-up to 100ml
- Filter sterilize
- Store at 4°C

BUFFER A (SALTING-OUT PROTOCOL)

- 10mmol/L Tris (Boehringer Mannheim) 1.21g/L
- 100mmol/L NaCl (Saarchem) 5.84g/L
- 10mmol/L EDTA (Boehringer Msnnheim) 3.72g/L
- Add less than 1L of water
- Allow to dissolve
- Adjust to pH 8
- Make-up to 1L
- Autoclave

CHLOROFORM:ISOAMYL ALCOHOL (24:1)

- 960ml chloroform (Saarchem / Labchem)
- 40ml isoamyl alcohol (Merck)
- Decant and mix in the fumehood

70% ETHANOL

- 70ml absolute ethanol (Analar)
- 30ml best quality water
- Mix thoroughly and store in dark bottle

10mg/ml ETHIDIUM BROMIDE STOCK

- 0.1g ethidium bromide (Sigma)
- 10ml best quality water
- Store at 4°C

1M ETHYLENEDIAMINE TETRAACETIC ACID (EDTA) STOCK

- 37.22g $Na_2EDTA.2H_2O$ (Boehringer Mannheim)
- Add 80ml of water
- Adjust pH 7.5 or 8 with NaOH
- Make-up to volume (100ml)
- Autoclave

FICOLL LOADING DYE

- 50g sucrose(50%) (Saarchem)
- 10ml EDTA, pH 7 (50mM)(Boehringer Mannheim)
- Bromophenol blue (0.1%) (Merck)
- 10g Ficoll (10%)
- Make-up to 100ml with best quality water

FIXING SOLUTION

- 15% methanol (150ml/L) (Saarchem)
- 15% acetic acid (50ml/L) (Labchem)
- Make-up to 1L with distilled water

20x GLYCEROL TOLERANT BUFFER

- 108g Tris-base (Boehringer Mannheim)
- 36g taurine (USB)
- 2g $Na_2.EDTA.2H_2O$ (Boehringer Mannheim)
- Make-up to 500ml
- Filter sterilise or autoclave

8% GLYCEROL TOLERANT SEQUENCING GEL

- 42g urea (Promega)
- 20ml 40% bis-acrylamide stock
- 5ml 20x glycerol tolerant buffer
- 35ml best quality water

- Dissolve
- Make-up to 100ml
- Add 600µL 0.1% ammonium persulphate (BIORAD) and 25µL TEMED (Promega), stir
- Pour immediately before polymerization begins

1M NaCl

- 58.44g sodium cloride (Saarchem)
- 1L water

- Allow to dissolve
- autoclave

10% Sodium Dodecyl Sulfate (SDS)

- 10g SDS (Boeringer Mannheim)

- Make-up to 100ml with best quality water
- Do not autoclave

SATURATED NaCl (6mol/L)

- 35g NaCl (Saarchem)
- Make-up to 100ml with best quality water
- Do not autoclave

10x TBE

- 108g Tris-base (Boehringer Mannheim)
- 55g boric acid (Sigma)
- 7.44g EDTA (Boehringer Mannheim)
- Make-up to 1L with water
- Autoclave

TE BUFFER (pH 7.4)

- 10mmol/L Tris (pH 7.4) 1.21g/L (Boehringer Mannheim)
- 1 mmol/L EDTA (pH 8.0) 0.273g/L (Boehringer Mannheim)
- Add 800ml of best quality water
- pH to 8.0
- Make-up to 1L with best quality water
- Autoclave

1M TRIS

- 121.1g tris (Boeringer Mannheim)
- 1L water
- Allow to dissolve
- Autoclave

Appendix C: PUBLICATION

High Prevalence of 1762^T 1764^A Mutations in the Basic Core Promoter of Hepatitis B Virus Isolated From Black Africans With Hepatocellular Carcinoma Compared With Asymptomatic Carriers

MARINA BAPTISTA, ANNA KRAMVIS, AND MICHAEL C. KEW

The purpose of this study was to identify mutations in the basic core promoter and enhancer II region of the hepatitis B virus (HBV) that might cause the HBV e antigen (HBeAg)-negative phenotype and contribute to hepatocarcinogenesis in black African carriers of the virus. The basic core promoter/enhancer II overlaps with the X gene. HBV DNA from serum of 47 asymptomatic carriers and 50 patients with hepatocellular carcinoma and from 28 tumor and 10 nontumor liver tissues was amplified and sequenced directly. That part of the enhancer II region not overlapping the basic core promoter was completely conserved in all samples. Missense mutations at nucleotides 1809 and 1812 in the basic core promoter were found in 80% of all sequences and may represent wild-type sequence in Southern African isolates. Nucleotide and amino acid divergences were higher in the basic core promoter of hepatocellular carcinoma patients when compared with asymptomatic carriers ($P < .0001$). This applied particularly to the paired 1762 adenine to thymine (1762^T) and 1764 guanine to adenine (1764^A) missense mutations, the prevalence of which was 66% in patients with hepatocellular carcinoma compared with 11% in asymptomatic carriers ($P < .0001$). There was no association between the presence of 1762^T 1764^A and HBeAg negativity, although these mutations suppressed HBeAg titers in HBeAg-positive patients. Suppression of HBeAg expression as well as alteration of the amino acid sequence of the X protein may play a role in hepatocarcinogenesis. (HEPATOLOGY 1999;29:946-953.)

Black Africans who are symptomless carriers of the hepatitis B virus (HBV) usually seroconvert from HBV e antigen (HBeAg) positivity to negativity after a relatively short time.[1] The carrier state is established in the first few years of life,[2] and by early adulthood between 1% and 14% only of black carriers remain HBeAg positive compared with 40% or more among ethnic Chinese, another population in which the virus is endemic.[1-4] Why black African carriers seroconvert earlier is not known. Nucleotide sequencing of the precore region of HBV isolates from a number of countries has shown the 1896 stop codon mutation to be a frequent cause of the HBeAg-negative phenotype,[5,6] with other nonsense or frame-shift mutations accounting for a small proportion (reviewed in Miyakawa et al.[7]). However, these mutations are seldom present in black African carriers in Southern Africa[8,9] and our focus has shifted to the X open reading frame.

The X open reading frame encodes the X protein that has transactivating activity (reviewed in Koike and Takada[10]) and contains the basic core promoter (BCP)/enhancer II complex. The BCP, mapping between nucleotides 1742 and 1849, controls the transcription of both precore messenger RNA (mRNA), which codes for the protein that is the precursor of the e antigen, and pregenomic RNA (pgRNA), which controls HBV replication.[11] A pair of mutations in the BCP that is associated with a reduced level of HBeAg expression was first described in Japanese patients[12-14]: an adenine (A) to thymine (T) transversion at position 1762 together with a guanine (G) to A transition at 1764 in the second AT-rich region of the BCP are often present in patients with chronic hepatitis B[15-20] and fulminant hepatitis B,[14-16,21-23] and less often in asymptomatic carriers (ASCs),[17] immunosuppressed patients,[24] and carriers without serological HBV markers.[25,26] These two mutations have also been reported in HBV DNA from sera[17] and tumor and liver tissue from patients with HBV-related hepatocellular carcinoma (HCC),[27] although the significance of this finding is yet to be determined.

The purpose of this study was to examine the nucleotide sequence of the BCP and the adjacent enhancer II region for mutations that might result in the early onset of the HBeAg-negative phenotype in black African carriers of HBV and to investigate a possible role for these mutations in hepatocarcinogenesis in this population.

MATERIALS AND METHODS

Serum and Tissue Samples. Serum samples were obtained from 111 hepatitis B surface antigen (HBsAg)-positive Southern African blacks: 52 were ASCs with normal serum alanine transaminase levels, and 59 were patients with HCC. The ASC and HCC patients were comparable in age. The serum of 15 of the ASCs was HBeAg positive and that of 37 was HBeAg negative/anti-HBe positive. Of the HCC patients, the serum of 25 was HBsAg positive and that of 34 was HBsAg negative/anti-HBe positive. Serum samples were stored

Abbreviations: HBV, hepatitis B virus; HBeAg, HBV e antigen; BCP, basic core promoter; mRNA, messenger RNA; pgRNA, pregenomic RNA; ASC, asymptomatic carrier; HCC, hepatocellular carcinoma; HBsAg, HBV surface antigen; HBcAg, HBV core antigen (core protein); PCR, polymerase chain reaction.

From the Medical Research Council/CANSA/University Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa.

Received June 9, 1998; accepted November 19, 1998.

Supported by a grant to A.K. from H.E. Griffin Cancer Trust. M.B. is a recipient of postgraduate bursaries from the Foundation for Research and Development, the Poliomyelitis Research Foundation, and H.E. Griffin Cancer Trust.

Address reprint requests to: Michael C. Kew, M.D., Department of Medicine, Witwatersrand University Medical School, 7 York Road, Parktown, 2193, Johannesburg, South Africa. E-mail: 014anna@chiron.wits.ac.za; fax: (27) 0 11-643-4318.

0270-9139/99/2903-0045$3.00/0

at −70°C until analyzed. Tumor and corresponding nontumor liver tissues were obtained at necropsy or laparotomy from 10 patients with HCC. Tumor tissue alone was obtained from a further 19 patients. Seven of the tumor/nontumor tissue pairs were obtained from patients whose serum was HBsAg positive/HBeAg negative and 3 from those who were anti-HBs/anti-hepatitis B core (HBc) positive. Serum in the majority of the patients (16 of 19) from whom tumor tissue only was obtained was HBsAg positive/ HBeAg negative, in 2 patients anti-HBs/anti-HBc positive, and in one patient anti-HBc positive alone. The tissues were snap-frozen in liquid nitrogen and stored at −70°C. Sera from healthy Southern African blacks without serological markers of HBV infection served as negative controls. Commercially available kits were used to detect HBV markers in serum (Abbott Laboratories, Chicago, IL).

DNA Extraction From Serum. DNA was extracted from serum using the QIAmp blood kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. A 200 µL aliquot was incubated with 25 µL Qiagen protease and 200 µL buffer AL at 70°C for 10 minutes. The sample was then incubated at 95°C for 15 minutes. The lysate was applied to a QIAmp spin column, centrifuged, and washed three times with buffer AW. DNA was eluted from the column with 50 µL best quality water.

DNA Extraction From Tissue. DNA from tumor and nontumor tissue was extracted by a modified salting out procedure.[28] One hundred milligrams of tissue was cut up and suspended in 5 mL buffer A (10 mmol/L Tris, 100 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid, pH 8), 200 mg/mL proteinase K, and 10% sodium dodecyl sulfate. The tissue pulp was digested for 2 to 3 hours at 37°C with continuous mixing. Two milliliters of saturated NaCl (approximately 6 mol/L) was added to the sample which was shaken gently for 15 minutes at room temperature. The sample was centrifuged and the supernatant transferred to a clean polypropylene tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, and the sample was mixed gently for 15 minutes at room temperature. The sample was then centrifuged at 3,500 rpm for 10 minutes at 8°C. The supernatant was transferred to a clean polypropylene tube, an equal volume of chloroform was added, and the solution was mixed gently for 5 minutes. The sample was centrifuged and the supernatant transferred to a clean plastic tube. Isopropanol was added gradually until the DNA precipitated out of solution. The precipitated DNA strands were removed with a pipet, washed for 2 minutes in 70% ice-cold ethanol, and resuspended in 200 µL TE buffer.

Polymerase Chain Reaction. The core promoter/enhancer II region was amplified by the polymerase chain reaction (PCR), using primers mapping nucleotides 1661 to 1921 (Table 1). PCR was performed in 25 µL and 50 µL final reaction volumes for first and second rounds, respectively. The reaction mixture consisted of 0.01 U/µL DynaZyme DNA polymerase (Version 2.0; Finnzymes OY, Espoo, Finland), 200 µmol/L of each of the dNTPs, 1 µmol/L of each of the primers, and 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.8), 1.5 mmol/L $MgCl_2$, and 0.1% triton-X 100. Both rounds of PCR were performed in a programmable thermal cycler (Perkin Elmer, Norwalk, CT). The cycle profile for the first round was 94°C for 30 seconds for denaturation, 60°C for 50 seconds for annealing, and 72°C for 50 seconds for polymerization, for 40 cycles, followed by a final extension step of 10 minutes at 72°C. For the second round of the nested PCR, the first round amplicon was diluted (1:100) and 5 µL of this was reamplified using the inner primers. The cycling conditions were as stated previously except that annealing was performed at 45°C for 30 seconds. Best quality water instead of DNA and positive and negative controls were included in both rounds of PCR. Sera positive for HBsAg, HBeAg, and HBV DNA by slot/blot hybridization were used as positive controls and sera from healthy blacks lacking all HBV markers as negative controls. To avoid cross contamination and false-positive results, the precautions and procedures suggested by Kwok and Higuchi[29] were strictly adhered to. DNA extraction, PCR, and electrophoresis were performed in physically separated venues.

Detection of Amplified Products. A 5-µL aliquot of the amplified product was electrophoresed on a 2% agarose gel. After nested PCR, a 260-bp band was visualized under ultraviolet light after ethidium bromide staining.

Nucleotide Sequencing of PCR Products. PCR products were sequenced directly by the dideoxy termination method using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) with modifications.[30] PCR products were sequenced in forward and reverse directions using the inner PCR primers. In a few instances the 1897R primer was used instead of the 1921R primer (Table 1).

Statistics. χ^2, Fisher exact, and Student's *t* tests were used to analyze the data.

TABLE 1. Oligonucleotide Primers Used in the Study

Primer	Sequence	Position*	Size (bp)†
Outer primers			
1622(+)	5'GAACGCCCATCAGATCCTGC3'	1622-1641	344
1966R(−)	5'GTCAGAAGGCAAAAACGAGAG3'	1966-1946	
Inner primers			
1661(+)	5'GACTCTTGGACTCTCAGC3'	1661-1678	260
1921R(−)	5'TTTATACGGGTCAATGTC3'	1921-1904	
Sequencing primer			
1897R	5'CCAAAGCCACCCAAGGCACAG-CTTGGAGGCTT3'	1897-1866	

Abbreviations: +, sense; −, antisense.

*Nucleotide position of HBV *adw* (GenBank accession no. V00866) where the EcoRI cleavage site is position 1. To achieve optimum melting temperature (Tm) we made one nucleotide change (C → T) at position 14 of primer 1661(+) (coincidentally changing it to *ayw* sequence) and one nucleotide change (T → C) at position 7 of primer 1921R(−).

†Size of the PCR product in base pairs.

RESULTS

Specific bands of the X/precore region of expected size (260 bp) were detected in agarose gels after amplification by nested PCR in all HBeAg-positive sera from 15 ASC and 25 HCC patients. A lower proportion of HBeAg-negative sera was HBV DNA positive by nested PCR: 32 of 37 (86%) ASC and 25 of 34 (73.5%) HCC serum samples. Twenty eight of 29 tumor tissues and all of 10 nontumor tissues were PCR positive.

Sequence analysis was only possible in samples with amplifiable X gene without deletions or insertions. HBV DNA amplified from both serum samples and liver tissue was successfully sequenced in the region 1683 to 1849 which includes part of the enhancer II (1685-1773) and the BCP (1742-1849). Furthermore, this region is translated into the carboxyl-terminus of the X protein and the amino terminus of the HBeAg precursor.

Enhancer II Sequences. The sequence of the enhancer II region not overlapping with the BCP, i.e., 1685 to 1741, was found to be completely conserved when aligned to sequence EMBL X70185 (subtype *adw2*[31]). There was no difference in this region according to the HBeAg–anti-HBe status or carrier type (ASC vs. HCC).

BCP Sequences. In total, 42 different nucleotide sequence patterns of the BCP region (1742-1849), when aligned to EMBL X70185, were detected in HBV extracted from sera of ASC and HCC patients and tumor and nontumor liver samples (Figs. 1-3). Highlighted sequences were unique to the serum of a particular patient population or tissue sample. In HBV from serum samples, missense mutations were found in 40% of ASC and 80% of HCC patients (not significant).

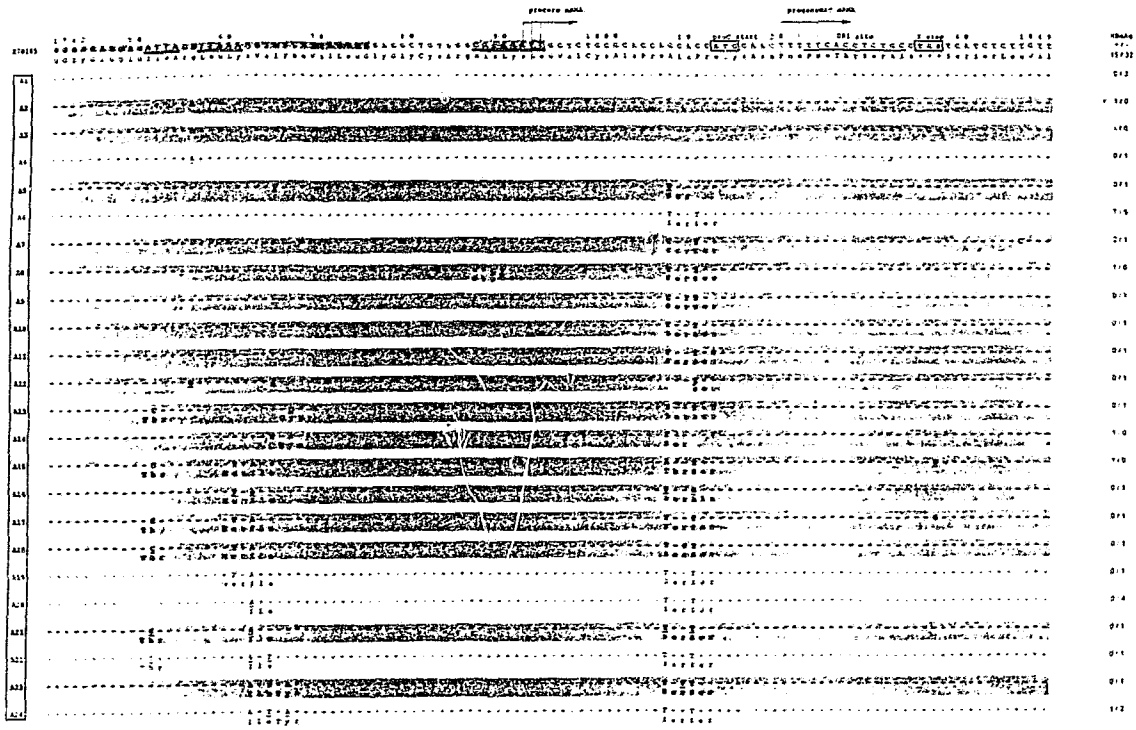


FIG. 1. Nucleotide sequence and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from ASCs aligned to sequence EMBL X 70185 (shown in full). Transcription factor binding sites[1,33,34] are *highlighted* and TA-rich regions are *underlined with a double line*. Twenty-four different sequences were found in 47 ASCs. *Highlighted sequences were found only in sera of ASCs. Numbers on the right* are the number found in HBeAg-positive (+) and HBeAg-negative (−) carriers. Substitutions that occurred together with wild-type nucleotide are *underlined*. ***Nonsense mutation.

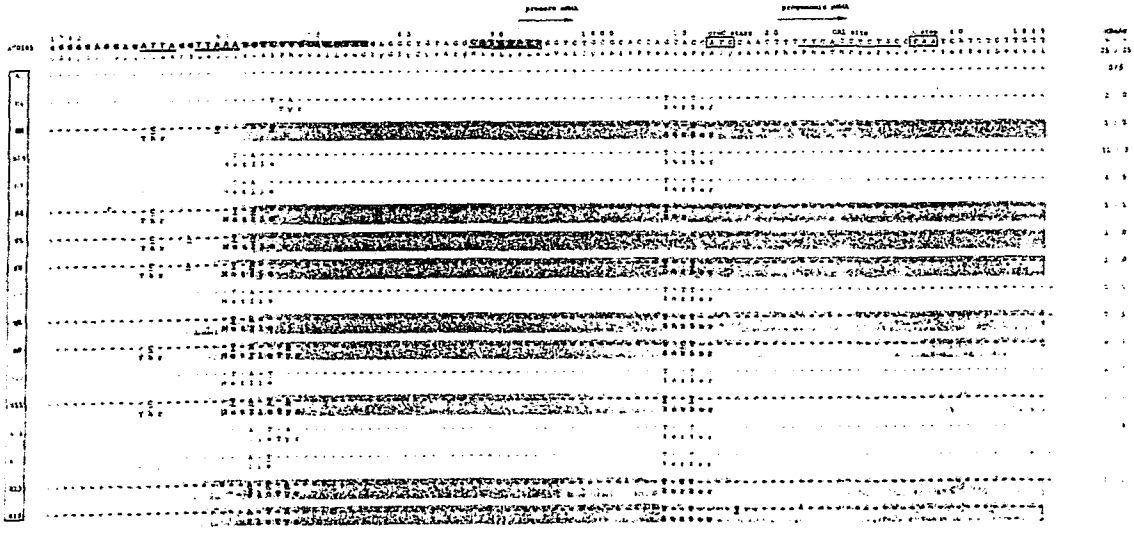


FIG. 2. Nucleotide sequence and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from HCC patients aligned to sequence EMBL X 70185 (shown in full). Transcription factor binding sites[1,33,34] are *highlighted* and TA-rich regions are *underlined with a double line*. Seventeen different sequences were found in 50 HCC patients. *Highlighted* sequences were found only in sera of HCC patients. *Numbers on the right* are the number found in HBeAg-positive (+) and HBeAg-negative (−) patients. Substitutions that occurred together with wild-type nucleotide are *underlined*.

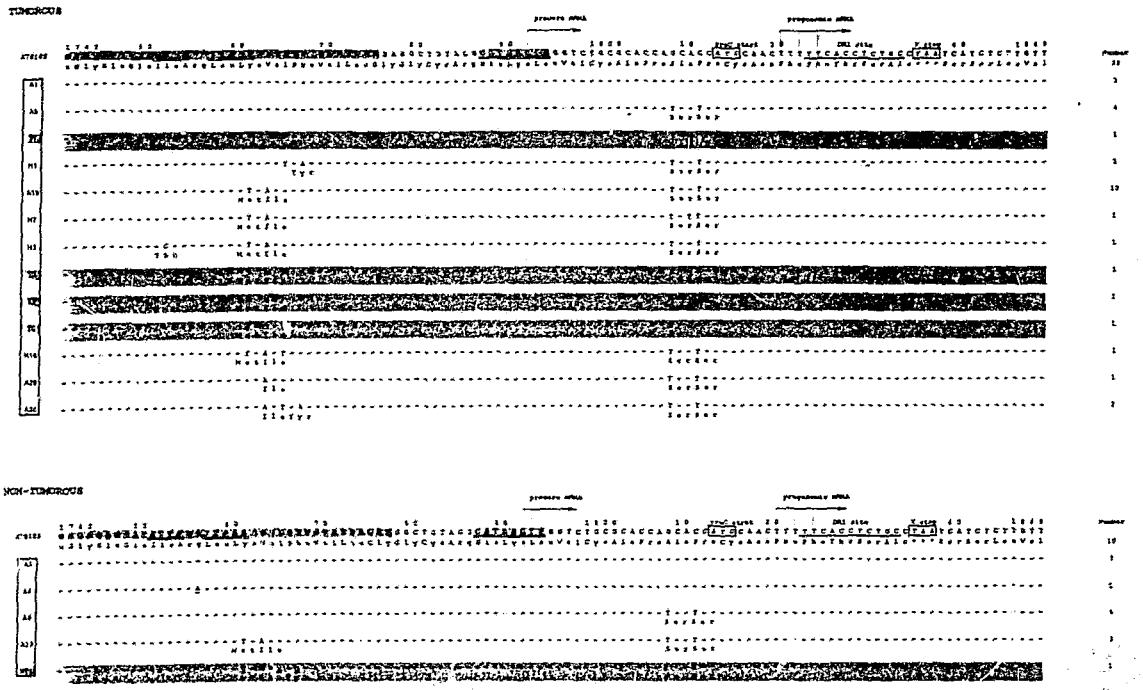


FIG. 3. Nucleotide sequence and deduced amino acid sequence of the BCP (1742-1849) of HBV DNA isolated from liver tissue aligned to sequence EMBL X 70185 (shown in full). Transcription factor binding sites[31,33,34] are *highlighted* and TA-rich regions are *underlined with a double line*. The HBV BCP sequences from 29 tumor and 10 nontumor liver samples are shown in *top* and *bottom* sections, respectively. *Highlighted* sequences were found only in liver tissue. Substitutions that occurred together with wild-type nucleotide are *underlined*.

Seventy-one percent of tumor tissues compared with 44% of the nontumor tissues samples had missense mutations (n.s.). Table 2 compares nucleotide and amino acid sequence divergence in BCP and precore regions of ASC and HCC patients. Both nucleotide and amino acid sequences were better conserved in the precore region[8,9] compared with the BCP. Percent nucleotide and amino acid sequence divergence was significantly higher in the BCP compared with the precore region ($P < .0001$). In the BCP both nucleotide and amino acid sequence divergence were significantly higher in virus extracted from HCC patients compared with ASCs ($P < .0001$).

In five patients the BCP sequences of HBV DNA were found to be identical in both tumor and nontumor tissues. In

TABLE 2. Nucleotide and Amino Acid Sequence Divergence in the BCP and Precore Regions of HBV Isolates From Sera of ASC and HCC Patients

	Basic Core Promoter			Precore Region*		
		% Nucleotide Divergence	% Amino Acid Divergence		% Nucleotide Divergence	% Amino Acid Divergence
	n	Mean ± SD	Mean ± SD	n	Mean ± SD	Mean ± SD
ASC						
Total	47	2.7 ± 1.5†‡	6.8 ± 3.9†§	57	1.5 ± 1.3	2.2 ± 2.6
HBeAg positive	15	2.4 ± 1.3	6.3 ± 3.6	16	1.5 ± 2.2	1.5 ± 2.2
HBeAg negative	32	2.8 ± 1.6	7.0 ± 4.0	41	1.6 ± 1.3	2.3 ± 2.7
HCC						
Total	50	4.0 ± 1.5†‡	10.7 ± 4.0†§	61	1.5 ± 1.3	2.2 ± 2.7
HBeAg positive	25	4.2 ± 2.0	11.3 ± 5.0	23	1.3 ± 1.3	1.5 ± 2.0
HBeAg negative	25	3.8 ± 2.0	9.9 ± 5.3	38	1.5 ± 1.3	2.7 ± 3.0
All						
Total	97	3.3 ± 1.6	8.8 ± 4.4	118	1.5 ± 1.3	2.2 ± 2.6
HBeAg positive	40	3.5 ± 1.3	9.5 ± 3.7	39	1.5 ± 1.2	1.5 ± 2.0
HBeAg negative	57	3.2 ± 1.8	8.3 ± 4.8	80	1.5 ± 1.3	2.5 ± 2.8

*These results are derived from previous studies (Kramvis et al., 1997, 1998).
†These results are statistically different using the Students *t* test.
‡$P < .0001$ comparing nucleotide divergence in BCP of HBV isolates from ASC and HCC patients.
§$P < .0001$ comparing amino acid divergence in BCP of HBV isolates from ASC and HCC patients.

another five, tumor tissues had sequences different from those found in the corresponding nontumor tissues. The majority (4/5) of the latter tumor-derived sequences had missense mutations, whereas those derived from the nontumor tissues had wild-type sequences (Table 3, Fig. 3). However, there was no significant difference between nucleotide and amino acid divergence in tumor and nontumor tissue. Both tumor and nontumor liver tissue had a nucleotide divergence of 3%, whereas tumor tissue had an amino acid divergence of 8.6% and nontumor tissue of 7%.

The BCP sequences obtained from both sera and tissues were characterized by two very well conserved regions: 1770 to 1808 and 1813 to 1849; and two hypervariable regions: 1753 to 1769 and 1809 to 1812. In a GenBank search sequence m57663 (subtype *adw*; isolated from a Philippino[32]) was found to have the same mutations at 1809 and 1812. These missense mutations, 1809 (G→T; alanine to serine) and 1812 (C→T; proline to serine) may be characteristic of Southern African HBV isolates because they were found in 80% of the sequences.

The most common changes in the first hypervariable region 1753 to 1769 were the missense mutations at 1762 (A→T; lysine to methionine) and 1764 (G→A; valine to isoleucine). The 1762^T was never found to occur in the absence of 1764^A, whereas 1764^A did occur alone. Table 4 compares the frequency of these single and double BCP mutations in the different patient populations and in the HBeAg-positive and HBeAg-negative serum samples. There was no significant difference in the incidence of the double 1762^T 1764^A mutant between HBeAg-negative and HBeAg-positive patients, nor was the incidence of the double mutation affected by the genotype as determined from sequences of the precore region. However, when HBeAg titers were compared in HBeAg-positive ASCs and HCC patients with wild-type precore region, the levels were significantly lower in patients carrying the virus with the 1762^T 1764^A double mutants (Fig. 4), *i.e.*, 3.0 in the mutant versus 7.8 in the wild-type ($P < .0001$). This equates to a 60% reduction in HBeAg expression. The frequency of the double 1762^T 1764^A mutant was significantly higher in HCC patients when compared with ASCs (Table 4) ($P < .0001$). Sequence A19 was the sequence most commonly found in sera and tissue of HCC patients (Figs. 2 and 3) and only in 1 ASC (Fig. 1). Moreover, sequence H3, which in addition to the 1762^T 1764^A mutation has a mutation at position 1753, was only found in sera and tissue of HCC patients and not in ASCs.

TABLE 3. Sequence Analysis of HBV DNA Extracted From Tumor and Nontumor Liver Samples

Group	Status	#	Sequence Analysis: Tumor	Sequence Analysis: Nontumor
A	HBsAg positive/ HBeAg negative	L1	Missense (A19)*	Missense (A19)*
		L2	Wild-type (A6)	Wild-type (A6)
B	HBsAg positive/ HBeAg negative	L3	Wild-type (A6)	Wild-type (A6)
		L4	Missense (A19)*	Missense (A19)*
		L5	Missense (H7)*	Wild-type (A6)
		L6	Missense (A20)†	Wild-type (A4)
		L7	Missense (A19)*	Wild-type (A6)
		L8	Missense (H3)*	NA
		L9	Missense (H1)	NA
		L10	Missense (A19)*	NA
		L11	Missense (T4)*	NA
		L12	Wild-type (T1)	NA
		L13	Missense (H10)*	NA
		L14	Missense (A24)†	NA
		L15	Wild-type (A1)	NA
		L16	Wild-type (A1)	NA
		L17	Wild-type (A1)	NA
		L18	Missense (A19)*	NA
		L19	Missense (A24)†	NA
		L20	Missense (T2)*	NA
		L21	Missense (A19)*	NA
		L22	Missense (H1)	NA
C	anti-HBs positive/ anti-HBc negative	L23	Missense (A19)*	Missense (A19)*
		L24	Missense (A19)*	Wild-type (A1)
		L25	Wild-type (A6)	Missense (NT1)*
		L26	Wild-type (A6)	NA
		L27	Missense (A19)*	NA
		L28	Missense (T3)*	NA

NOTE: Numbers in parentheses refer to sequence numbers in Fig. 3.
Abbreviation: NA, tissue not available.
*With 1762^T 1764^A.
†With 1764^A.

TABLE 4. Frequency of BCP Mutations in HBV Isolates From ASC and HCC Patients

Sample Patients	#	1762^T 1764^A Mutant	1764^A	Total
Sera				
ASC	47	5 (11%)*†	10 (21%)	15 (32%)*‡
HBeAg positive	15	1 (7%)	1 (7%)	2 (14%)
HBeAg negative	32	4 (13%)	9 (28%)	13 (41%)
HCC	50	33 (66%)*†	9 (18%)	42 (84%)*‡
HBeAg positive	25	20 (80%)	2 (8%)	22 (88%)
HBeAg negative	25	13 (52%)	7 (28%)	20 (80%)
Total	97	38 (39%)	19 (20%)	57 (59%)
HBeAg positive	40	21 (53%)	3 (8%)	24 (61%)
HBeAg negative	57	17 (30%)	16 (28%)	33 (58%)
Tissues				
Tumor	28	15 (54%)	3 (11%)	18 (65%)
Nontumor	10	4 (40%)	0 (0%)	4 (40%)

*These results are statistically different using the χ^2 test.

†$P < .0001$ comparing the frequency of 1762^T1764^A mutant in HCC vs. ASC. This significant difference also holds when sequences with mixed wild-type/mutant were excluded.

‡$P < .0001$ comparing the frequency of 1762^T1764^A and 1764^A mutant in HCC vs. ASC. This significant difference also holds when sequences with mixed wild-type/mutant were excluded.

DISCUSSION

The economy with which HBV uses its genome is very evident in the region sequenced in this study. The DNA spanning nucleotides 1685 to 1849 has a dual function. First, it contains the BCP and part of enhancer II, which are important in the control of transcription of both precore mRNA and pgRNA.[35] Precore mRNA is translated into the precore-core fusion protein that is post-translationally modified into soluble HBeAg, whereas pgRNA is translated into core and polymerase proteins and is reverse transcribed into minus-strand DNA. Second, this region encodes the carboxyl-terminus of the X protein, which has transactivating functions. Mutations in this region could therefore influence HBeAg production and viral replication as well as the amino acid sequence of the X protein.

The precore region in Southern African isolates of HBV studied previously in the same sera,[8,9] showed significantly less nucleotide and amino acid divergence than did the BCP

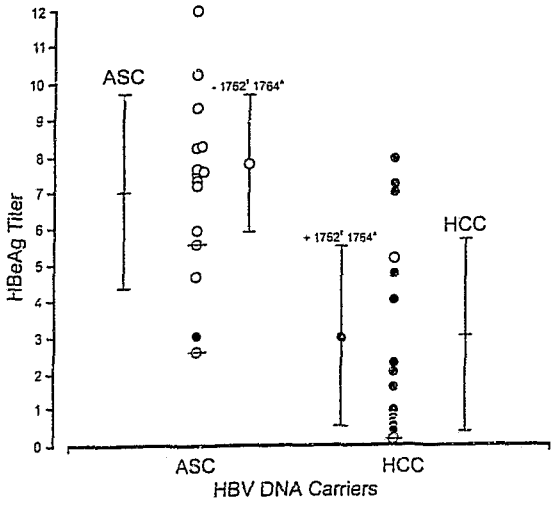


FIG. 4. Comparison of HBeAg titers between HBeAg-positive ASC and HCC patients with and without 1762^T 1764^A. HBeAg titers as determined by radioimmunoassay of serum samples. A level of 1.0 corresponds to 1,000 cpm. *Black and white points* are for samples with and without 1762^T 1764^A, respectively. The *white point with slash is a sample with* 1764^A *without 1762*. The serum HBeAg titers were significantly lower in patients with 1762^T 1764^A than those without ($P < .0001$). The difference in HBeAg titers between HBeAg-positive ASCs and HCC patients were also significantly different ($P < .0001$). The mean and standard deviation of titers from each group are shown.

This was true in both ASCs and patients with HCC. Moreover, the nucleotide and amino acid divergences in the precore region were the same in ASCs and HCC patients, whereas in the BCP they were significantly higher in the HCC patients ($P < .0001$) (Table 2). Because the nucleotide and amino acid substitution rates are generally similar in the X and core regions,[36] the higher nucleotide and amino acid divergence rates in the BCP in HCC sera might indicate a selection of virus with missense mutations in this region. In fact, 80% of HCC-derived sequences had missense mutations compared with 40% in ASCs. There appeared to be no differences in the sequence divergences between tumor and nontumor tissue. However, the number of nontumor tissues was too few to reach any firm conclusions.

In agreement with other studies,[37,38] two highly conserved regions were distinguished in the nucleotide sequences of the BCP of HBV isolated from both ASCs and HCC patients. This is to be expected because these regions have been shown to be crucial in regulating transcription and replication. In the first region (1770 to 1808 [Figs. 1-3]), mutations were found in only 2 of 130 sequences and these occurred as mixed populations with wild-type sequence. The latter must have a compensatory effect, overcoming the potentially lethal effect of the mutation and allowing viral replication to proceed. This region contains sequences corresponding to the Kunitz domain of serine protease inhibitors[10] and has been shown to be essential for the transactivating function of X protein.[10,39] The precore mRNA initiation site (1793-1795) also maps within this region.[11] The second conserved region contains the most crucial element of the BCP, namely the initiation site of pgRNA transcription (1821-1825),[11] as well as coding for the carboxy-terminal of the X protein. The overlap of this region with the 5′ end of the precore open reading frame containing direct repeat 1 is an additional reason for the low sequence divergence.

Mutations at positions 1809 and 1812 have previously been reported to occur only occasionally: in a Philippino man,[32] in 1 out of 29 HBV strains from 14 different countries,[37] and in chronic hepatitis patients after orthotopic liver transplantation.[40] In the present study these mutations were found in 80% of all sequences. This suggests that they represent wild-type sequence in these isolates and that they do not interfere with viral replication, although they disrupt the Watson-Crick base pairs in the secondary structure of the pgRNA around direct repeat 1.[41] Furthermore, they might not affect the function of the X protein, notwithstanding that they are missense mutations converting the hydrophobic, larger alanine and proline to the smaller and polar serine.

The missense mutations detected most often in the BCP of Southern African isolates were the 1762^T and 1764^A in tandem and 1764^A alone. 1762^T alone was not found (Figs. 1-3, Table 4). These mutations have rarely been reported to occur alone in previous studies, whereas the double mutant has been described in ASCs[17] and patients with chronic hepatitis,[15-20] fulminant hepatitis,[14-16,21-23] and HCC.[17,27] The frequency of 1764^A alone was similar in ASCs and patients with HCC, but the prevalence of 1762^T 1764^A was significantly higher in HCC patients ($P < .0001$). As ASCs in black Africans are the individuals in whom HCC develops, the appreciable step-up in incidence of 1762^T 1764^A from ASCs to patients with HCC suggests that the double mutation may play a role in HBV-induced hepatocarcinogenesis.

In agreement with some[16,17,23] but not other studies,[6,37,38] we did not find an association between 1762^T 1764^A and HBeAg negativity (Table 4). However, when we compared HBeAg-positive sera with the 1762^T 1764^A mutant virus with sera with wild-type virus, HBeAg titers were reduced by 60% in the patients carrying the mutant virus ($P < .0001$) (Fig. 4), a finding that confirms that of Takahashi et al.[17] and Kurosaki et al.[18] This would indicate an HBeAg-suppressive phenotype of the virus with the double mutation,[17] an observation supported by transfection studies showing reduced levels of precore mRNA and HBeAg secretion in the presence of the mutations.[42-46] The mutational hot spots 1762 and 1764 lie within the protein binding sequences of the BCP (Fig. 1). Liver-specific nuclear factor CCAAT/enhancer binding protein bind to this region to activate transcription.[33,34] Recently, suppressed HBeAg synthesis has been shown to result from decreased binding of liver-enriched factor to the TA-rich region spanning nucleotides 1758-1764.[44,45] Although we cannot exclude the possibility that the differences in HBeAg titer were caused by host differences between ASCs and HCC patients rather than differences in the virus, we consider this to be unlikely. The one ASC patient with the double mutation had reduced HBeAg titer (Fig. 4), and when the double mutation was found in virus from Southern African blacks with fulminant hepatitis, HBeAg titer was also reduced (unpublished observation).

In vitro studies have generated controversial results in regard to the effects of 1762^T 1764^A on viral replication. Using a reporter gene under a mutated promoter, Nishizono et al.[47] failed to detect a significant difference in promoter activity when comparing mutant and wild-type HBV. In transfection studies 1762^T 1764^A suppressed precore mRNA transcription and enhanced viral replication.[42-45] Buckwold et al.[45] attrib-

uted increased viral replication to enhanced encapsidation and not increased pgRNA synthesis, whereas Moriyama et al.[42,43] ascribed it to increased pgRNA transcription. On the other hand, Gunther et al.[46] found reduced precore mRNA transcription only and no increased viral replication in transfection studies using HuH7 cells. The fact that we and others have detected these mutants in viral isolates from sera of ASC and HCC patients, which have low HBV DNA titers (based on the fact that HBV DNA could be detected only by using nested PCR), argues against these mutations causing increased viral replication. Further support for this view is provided by the finding that DNA polymerase levels were significantly lower in patients with the double mutant virus.[18] Thus, the prevalence of the 1762^{T} 1764^{A} mutant virus over wild-type virus in HCC patients appears not to result from increased viral replication but may be secondary to decreased HBeAg expression. HBeAg shares epitopes with HBcAg[48] and this has been postulated to induce immune tolerance against itself or HBcAg or both antigens.[49,50] In the presence of reduced or absent serum HBeAg, HBcAg may be targeted directly by both the cellular and humoral immune systems[48] leading to necrosis of hepatocytes and liver damage.[22,51] HBcAg elicits a significantly more vigorous antibody response *in vivo* than HBeAg.[48] Liver damage is an important contributing factor in the development of HBV-related HCC.[52] This offers one possible mechanism by which 1762^{T} 1764^{A} may contribute to hepatocarcinogenesis.

1762^{T} 1764^{A} are nonsynonymous and could affect the structure of the X protein. This mutation converts amino acids 130 and 131 of the overlapping X coding region from lysine to methionine (1762, A → T, AAG → ATC) and valine to isoleucine (1764; G → A, GTC → ATC), respectively. Lysine is a charged amino acid, whereas methionine is hydrophobic, and this alteration could be the reason why the 1762 mutation is rarely found alone. On the other hand, because both valine and isoleucine are hydrophobic, the 1764 mutation on its own is better tolerated. These mutations fall outside the region essential for transactivation (codons 132 to 148) but within the loop formed by the disulphide linkage between cysteine-61 and cysteine-137.[53] The amino acid changes caused by these mutations in the presence of serine/serine caused by changes at nucleotides 1809/1812 could therefore alter the structure of the X protein and thus contribute to hepatocarcinogenesis.

Acknowledgment: The authors thank Dr. E. Song for supplying some of the sera used in this study.

REFERENCES

1. Dusheiko GM, Bowyer SM, Paterson A, Song E, Dibisceglie A, Kew MC. Clinical and serological events accompanying changes in hepatitis B viral replication: case reports. Liver 1985;5:77-83.
2. Botha JF, Ritchie MJJ, Dusheiko GM, Mouton HWK, Kew MC. Hepatitis B virus carrier state in black children in Ovamboland: role of perinatal and horizontal infection. Lancet 1984;1:1209-1212.
3. Stevens CE, Szmuness W. Vertical transmission of hepatitis B and neonatal hepatitis B. In: Bianchi L, Gerok W, Sickinger K, Stalder GA, eds. Virus and Liver Disease. Lancaster: MTP Press, 1980:285-292.
4. Kew MC, Kassianides C, Berger EL, Song E, Dusheiko GM. Prevalence of chronic hepatitis B virus infection in pregnant black women living in Soweto. J Med Virol 1987;22:263-268.
5. Carman WF, Zanetti AR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 1989;2:588-590.
6. Okamoto H, Tsuda F, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, et al. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol 1990;64:1298-1303.
7. Miyakawa Y, Okamoto H, Mayumi M. The molecular basis of hepatitis B e antigen (HBeAg)-negative infections. J Viral Hepatitis 1997;4:1-8.
8. Kramvis A, Bukofzer S, Kew MC, Song E. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of Southern African black adult carriers of the virus. HEPATOLOGY 1997;25:235-240.
9. Kramvis A, Kew MC, Bukofzer S. Hepatitis B virus precore mutants in serum and liver of Southern African blacks with hepatocellular carcinoma. J Hepatol 1998;28:132-141.
10. Koike K, Takada S. Biochemistry and functions of hepatitis B virus X protein. Intervirology 1995;38:89-99.
11. Chen I-H, Huang C-J, Ting L-P. Overlapping initiator and TATA box functions in the basal core promoter of hepatitis B virus. J Virol 1995;69:3647-3657.
12. Moriyama K, Takada T, Tsutsomu Y, Fukada K, Ishibashi H, Niho Y, Maeda Y. Mutations in the transcriptional regulatory region of the precore and core/pregenome of a hepatitis B virus with defective HBeAg production. Fukuoka Acta Medica 1994;85:314-322.
13. Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 1994;68:8102-8110.
14. Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, Tsuda F, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241-248.
15. Horikita M, Itoh S, Yamamoto K, Shibayama T, Tsuda F, Okamoto H. Differences in the entire nucleotide sequence between hepatitis B virus genomes from carriers positive for antibody to Hepatitis B e antigen with and without active disease. J Med Virol 1994;44:96-103.
16. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618-1623.
17. Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, et al. The precore/core promoter mutant ($T^{1762}A^{1764}$) of hepatitis B virus: clinical significance and an easy method for detection. J Gen Virol 1995;76:3159-3164.
18. Kurosaki M, Enomoto N, Asahina Y, Sakuma I, Ikeda T, Tozuka S, Izumi N, et al. Mutations in the core promoter region of hepatitis B virus in patients with chronic hepatitis B. J Med Virol 1996;49:115-123.
19. Kanai K, Kako M, Aikawa T, Hino K, Tsubouchi H, Takehira Y, Iwabuchi S, et al. Core promoter mutations of hepatitis B virus for the response to interferon in e antigen-positive chronic hepatitis B. Am J Gastroenterol 1996;91:2150-2156.
20. Sterneck M, Gunther S, Santantonio T, Fischer L, Broelsch CE, Greten H, Will H. Hepatitis B virus genomes of patients with fulminant hepatitis do not share a specific mutation. HEPATOLOGY 1996;24:300-306.
21. Hasegawa K, Huang J, Rogers SA, Blum HE, Liang TJ. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J Virol 1994;68:1651-1659.
22. Kaneko M, Uchida T, Moriyama M, Arakawa Y, Shikata T, Gotoh K, Mima S. Probable implication of mutations in the X open reading frame in the onset of fulminant hepatitis B. J Med Virol 1995;47:204-208.
23. McMillan JS, Bowden DS, Angus PW, McCaughan GW, Locarnini SA. Mutations in the hepatitis B virus precore/core gene and core promoter in patients with severe recurrent disease following liver transplantation. HEPATOLOGY 1996;24:1371-1378.
24. Gunther S, Piwon N, Iwanska A, Schilling R, Meisel H, Will H. Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B virus from immunosuppressed patients with severe liver disease. J Virol 1996;70:8318-8331.
25. Preisler-Adams S, Schlayer H-J, Peters T, Hettler F, Gerok W, Rasenack J. Sequence analysis of hepatitis B virus DNA in immunologically negative infection. Arch Virol 1993;133:385-396.
26. Gotoh K, Mima S, Uchida T, Shikata T, Yoshizawa K, Irie M, Mizui M. Nucleotide sequence of hepatitis B virus isolated from subjects without serum anti-hepatitis B core antibody. J Med Virol 1995;46:201-206.
27. Hsia CC, Yuwen H, Tabor E. Hot-spot mutations in hepatitis B virus X gene in hepatocellular carcinoma. Lancet 1996;348:625-626.
28. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human infected cells. Nucleic Acids Res 1988;16:1215.
29. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989;339:237-238.

30. Casanova J-L, Pannetier C, Jaulin C, Kourilsky P. Optimal conditions for directly sequencing double-stranded PCR products with Sequenase. Nucleic Acids Res 1990;18:4028.
31. Preisler-Adams S, Schlayer HJ, Peters T, Korp R, Rasenack J. Complete nucleotide sequence of hepatitis B virus, subtype *adw2*, and identification of three types of C open reading frame. Nucleic Acids Res 1993;21:2258.
32. Estacio RC, Chavez CC, Okamoto H, Lingao AL, Reyes MT, Domingo E, Mayumi M. Nucleotide sequence of a hepatitis B virus genome of *subtype adw isolated from a Philippino: comparison with the reported* three genomes of the same subtype. J Gastroenterol Hepatol 1988;3:215-222.
33. Lopez-Cabrera M, Letovsky J, Hu K-Q, Siddiqui A. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: trans-activation and repression by CCAAT/enhancer binding protein. Proc Natl Acad Sci U S A 1990;87:5069-5073.
34. Zhang P, McLachlan A. Differentiation-specific transcriptional regulation of the hepatitis B virus nucleocapsid gene in human hepatoma cell lines. Virology 1994;202:430-440.
35. Yuh C-H, Chang Y-L, Ting L-P. Transcriptional regulation of precore and pregenomic RNAs of *hepatitis B virus. J Virol 1992;66:4073-4084.*
36. Orito E, Mizokami M, Ina Y, Moriyama EN, Kameshima N, Yamamoto M, Gojobori T. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. Proc Natl Acad Sci U S A 1989;86:7059-7062.
37. Kidd-Ljunggren K, Oberg M. Kidd AH. The hepatitis B virus X gene: analysis of functional domain variation and gene phylogeny using multiple sequences. J Gen Virol 1995;76:2119-2130.
38. Kidd-Ljunggren K, Oberg M, Kidd AH. Hepatitis B virus X gene 1751 to 1764 mutations: implications for HBeAg status and disease. J Gen Virol 1997;78:1469-1478.
39. Arii M, Takada S, Koike K. *Identification of three essential regions of* hepatitis B virus X protein for trans-activation function. Oncogene 1992;7:397-403.
40. Laskus T, Rakela J, Steers JL, Wiesner RH, Persing DH. Precore and contiguous regions of hepatitis B virus in liver transplantation for end-stage hepatitis B. Gastroenterology 1994;107:1774-1780.
41. Kidd AH, Kidd-Ljunggren K. A revised secondary structure model for the 3'-end of hepatitis B virus pregenomic RNA. Nucleic Acids Res 1996;24:3295-3301.
42. Moriyama K, Okamoto H, Tsuda F, Mayumi M. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology 1996;226:269-280.
43. Moriyama K. Reduced antigen production by hepatitis B virus harbouring nucleotide deletions in the overlapping X gene and precore-core promoter. J Gen Virol 1997;78:1479-1486.
44. Buckwold VE, Xu Z, Chen M, Yen TSB, Ou J-H. Effects of a naturally *occurring mutation in the hepatitis B virus basal core promoter on* precore gene expression and viral replication. J Virol 1996;70:5845-5851.
45. Buckwold VE, Xu Z, Yen TSB, Ou J-H. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. J Gen Virol 1997;78:2055-2065.
46. Gunther S, Piwon N, Will H. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653)→T, A(1762)→T and G(1764)→A mutations in the core promoter. J Gen Virol 1998;79:375-380.
47. Nishizono A, Hiraga M, Kohno K, Sonoda YT, Terao H, Fujioka T, Nasu M, et al. Mutations in the core promoter/enhancer II regions of naturally occurring hepatitis B virus variants and analysis of the effects on transcription activities. Intervirology 1995;38:290-294.
48. Milich DR, McLachlan A, Stahl S, Wingfield P, Thornton GB, Hughes JL, Jones JE. Comparative immunogenecity of hepatitis B virus C and E antigens. J Immunol 1988;141:3617-3624.
49. Milich DR, Jones JED, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance *in utero*? Proc Natl Acad Sci U S A 1990;87:6599-6603.
50. Carman W, Thomas H, Domingo E. Viral genetic variation: hepatitis B *virus as a clinical example. Lancet 1993;341:349-353.*
51. Brunetto MR, Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfarano A, Serra A, et al. Wild-type and HBeAg-minus hepatitis B viruses and course of chronic infection. Proc Natl Acad Sci U S A 1991;88:4186-4190.
52. Kew MC. Hepatitis B and C viruses and hepatocellular carcinoma. Clin Lab Med 1996;16:395-406.
53. Gupta A, Mai TK, Jayasuryan N, Chauhan VS. Assignment of disulphide bonds in the X protein (HBx) of hepatitis B virus. Biochem Biophys Res Commun1995;212:919-924.