

1.1.6 Genomic Organisation and Gene Products

The hepatitis B virus is the smallest DNA virus infectious to man [Francois *et al.*, 2001]. Its genome is approximately 3.2 kb, relaxed circular, partially double-stranded DNA (dsDNA), possessing a complete negative-strand and an incomplete positive-strand of variable length (1.7 to 2.8 kb) [Ngui *et al.*, 1999; Locarnini *et al.*, 2003]. Circularity of the genome is maintained by a 224 bp cohesive overlap demarcated by the 5' ends of the two DNA strands that contain 11 nucleotide direct repeats, DR 1 and DR 2 (*Figure 1.4*) [Seeger, 1994; Ngui *et al.*, 1999]. The negative-strand is not a complete circle as it is nicked within DR 1, leaving the 5' end free to covalently bind the terminal protein, or viral polymerase [Locarnini *et al.*, 2003]. The 5' end of the positive-strand is linked to an 18 base-long RNA oligonucleotide, which is derived from the 5' end of viral pregenomic RNA formed during replication [Seeger, 1994; Locarnini *et al.*, 2003].

Negative-strand DNA, the template for the transcriptional synthesis of viral mRNA, has four partly overlapping open reading frames (ORFs) – core, polymerase, X, and surface – that encode seven viral proteins (*Figure 1.4*) [Seeger, 1994]. Four mRNAs of different sizes are produced following transcription, and are subsequently modified by 3' polyadenylation and the addition of a 5' cap [Cattaneo *et al.*, 1983; Yokosuka *et al.*, 1986]. A single polyadenylation signal within the negative strand results in the termination of transcription at a common 3' end for all of the transcripts, while the 5' ends are variable as they are determined by the location of each of four HBV genomic promoters that bind host RNA polymerase II and initiate transcription [Ganem and Varmus, 1987]. The precore/pregenomic, preS1, preS2, and X promoters regulate the transcription of 3.5 kb, 2.4 kb, 2.1 kb, and 0.8 kb mRNA transcripts, respectively [Cattaneo *et al.*, 1983; Yokosuka

et al., 1986]. Additionally, two enhancers (Enhancer I and II) occur in the HBV genome and are able to greatly increase the rate of transcription [Su and Yee, 1992].

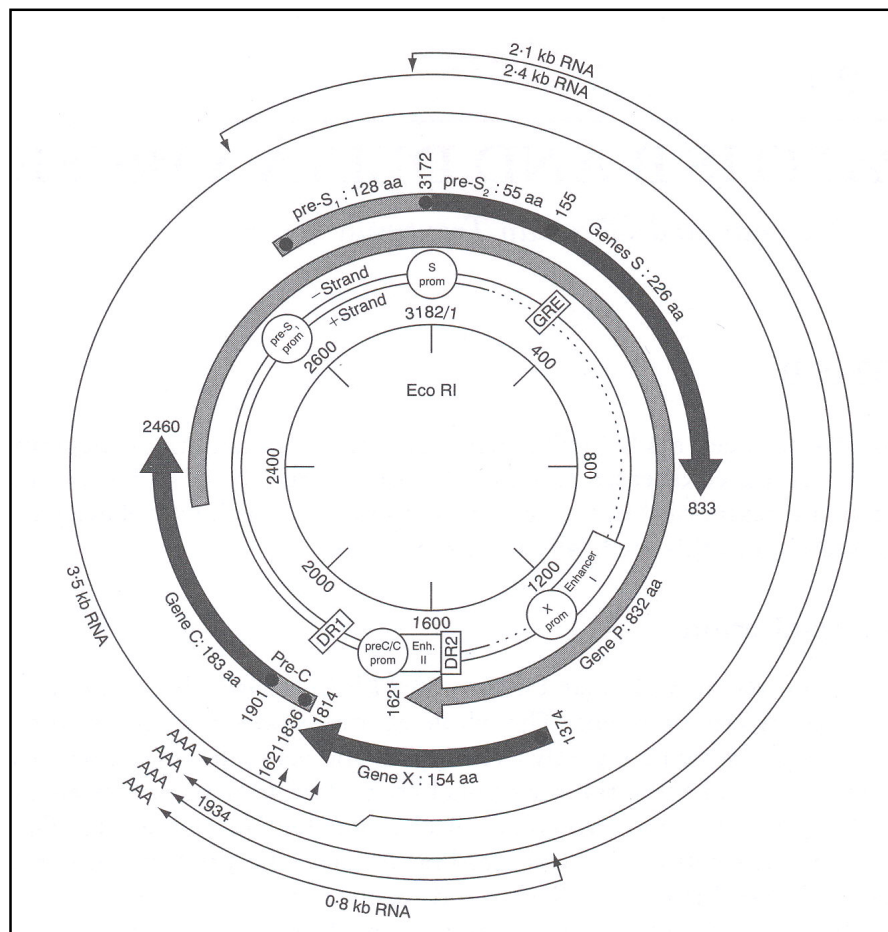


Figure 1.4 Schematic representation of the HBV genome. The numbered circle depicts the 3 182 bp partially double-stranded DNA. The lengths of the four mRNAs and seven proteins are shown, as are the promoter and enhancer elements [Carman and Trautwein, 1999].

1.1.6.1 Core Genes

The core (C) gene has two in-phase regions, the precore and core, which encode the hepatitis B e antigen (HBeAg) and the hepatitis B core antigen (HBcAg) or viral capsid protein, respectively, depending on the translational start codon (AUG) used [Carman, 1996].

Translated from the 3.5 kb pregenomic mRNA, the 21 kDa core protein consists of either 183, 185, or 195 amino acids (aa), depending on the genotype of the virus [Locarnini *et al.*, 2003]. A cytoplasmic phosphoprotein, HBcAg is the building block of the nucleocapsid core and, as such, is important in encapsidating the pregenomic RNA and viral polymerase protein [Carman, 1996]. The HBcAg protein has two distinct domains: the amino-terminal domain (residues 1 to 144) is required for the spontaneous self-assembly of the nucleocapsid; and the arginine-rich (positively charged) carboxy-terminus (from residue 150 onwards) mediates the binding of RNA and DNA, and is thus involved in viral encapsidation and DNA replication [Nassal and Schaller, 1993; Locarnini *et al.*, 2003]. The icosahedral shell of the nucleocapsid is composed of either 90 homodimers of core protein, with a T=3 symmetry, or 120 homodimers and a T=4 symmetry [Ponsel and Bruss, 2003]. In addition, 2 nm spikes that contain the major conformational B-cell epitope are present at the five fold axes of the capsid [Carman and Trautwein, 1999].

The precore sequence of the HBV genome is located 87 nucleotides upstream of the core. Initiating from the precore start codon, translation of the 3.5 kb precore mRNA produces a 25 kDa precore protein that consists of the entire core protein sequence and an additional 29 aa at the N-terminal end. An α -helix within the first 19 aa serves as a signal peptide

that allows the translocation of the precore protein into the lumen of the endoplasmic reticulum (ER) [Ou *et al.*, 1986]. Within the ER, the 19 aa are cleaved from the amino terminus by a host cell signal peptidase, resulting in a 22 kDa protein derivative [Jean-Jean *et al.*, 1989]. Cysteine and tryptophan residues within the remaining ten residues of the N-terminus prevent particle formation of this protein [Carman, 1996], which is transported through the ER and Golgi complex and further modified by cleavage of up to 34 aa from the carboxy-terminus [Jean-Jean *et al.*, 1989]. This ultimately results in the secretion into the serum of a heterogeneous population of 15 to 18 kDa proteins (~160 aa), serologically defined as HBeAg [Ganem, 1996]. The presence of HBeAg is conventionally indicative of high viral replication and infectivity [Francois *et al.*, 2001]. In addition to occurring in the serum, this antigenic protein is expressed on the surface of infected hepatocytes and is therefore a target for the immune defence mechanisms to destroy these cells [Francois *et al.*, 2001]. However, HBeAg also appears to induce immunological tolerance of T-helper cells against both itself and HBcAg [Milich *et al.*, 1990; Chen *et al.*, 2004], and its presence is therefore essential for the establishment of chronic infection and a resultant HBV carrier state [Carman, 1996; Francois *et al.*, 2001]. HBcAg and HBeAg are antigenically distinct [Seeger, 1994].

1.1.6.2 Polymerase Gene

As the longest ORF, the polymerase (P) gene spans almost 80% of the HBV genome and overlaps each of the other ORFs. Encoded by this gene and translated from the 3.5 kb pregenomic mRNA by a possible leaky scanning mechanism, the 90 kDa viral DNA polymerase, or reverse transcriptase, has between 834 and 845 aa [Seeger, 1994; Locarnini

et al., 2003]. The polymerase protein has at least four domains displaying enzymatic activities required for the synthesis of the viral DNA genome from the pregenomic RNA intermediate [Seeger, 1994].

The N-terminal domain of polymerase represents the terminal protein that is covalently linked to the 5' end of the negative-strand of viral DNA, via the hydroxyl group of tyrosine at residue 63 on the enzyme and the α -phosphate of the first nucleotide (G) of the DNA [Locarnini *et al.*, 2003]. The terminal protein therefore acts as a primer for reverse transcription of pregenomic RNA into DNA. The spacer or tether region is the second, intervening, domain and has no recognised function. Following the spacer, the third domain is an RNA- and DNA-dependent DNA polymerase, or reverse transcriptase, which contains five functional regions A, B, C, D, and E [Chen and Oon, 1999]. While region B is associated with attachment of the polymerase to the RNA template for reverse transcription, and region E is involved in initiation of the process, regions A, C, and D, bind dNTPs for DNA synthesis and comprise the catalytic centre of the enzyme that contains the 'classical' tyrosine-methionine-aspartate-aspartate (YMDD) motif common to reverse transcriptases [Locarnini, 2003; Torresi, 2002]. The fourth, C-terminal domain has ribonuclease (RNase) H activity that degrades the pregenomic RNA template immediately following negative-strand DNA synthesis. This domain additionally plays a role in viral RNA packaging, in optimising the priming of negative-strand DNA synthesis, and in the elongation of negative-strand DNA. Also occurring within the polymerase protein are two T-cell epitopes at amino acids 107 to 115 and 227 to 235 in the catalytic domains.

1.1.6.3 X Gene

The smallest of the HBV ORFs, the X gene encodes the 17 kDa, 146 to 154 amino acid-long HBx protein, which is translated from the 0.8 kb mRNA transcript [Ngui *et al.*, 1999; Moolla *et al.*, 2002; Locarnini *et al.*, 2003]. HBx is required for the establishment of HBV infection. Although this protein has not been fully characterised, it is known to behave as a transcriptional transactivator of a number of viral and cellular gene promoters [Locarnini *et al.*, 2003].

As a result of its pleiotropic effects, an important additional function of the HBx protein is as a cofactor in HBV-mediated carcinogenesis [Seeger, 1994; Carman and Trautwein, 1999; Locarnini, 2003]. It is thought that the transactivation activity of HBx may lead to alterations in cellular gene expression that contribute to transformation, thereby aiding in the development of HCC [Locarnini *et al.*, 2003]. The ability of HBx to bind and inactivate the transcription factor and tumour suppressor p53, and to abrogate p53-dependent apoptosis, further supports this protein's role in oncogenesis [Feitelson, 1994; Carman, 1996; Locarnini *et al.*, 2003].

1.1.6.4 Surface Genes

The surface (S) gene has three regions – preS1, preS2, and S – that possess three separate translational start sites (AUG) but share the same stop codon (UAA). In this way, three different sized proteins with a common carboxy-terminus are produced: a large (L) or preS1 protein, a middle (M) or preS2 protein, and a small protein or hepatitis B surface

antigen (HBsAg). Each of these is a transmembranous envelope protein, or surface antigen, that is covalently linked to the other viral envelope proteins by extensive intermolecular disulphide bonds between the cysteine residues of their S domains. The surface proteins exist in two forms, glycosylated and unglycosylated. Such N-linked glycosylation is necessary for virion, but not subviral particle, secretion [Stirk *et al.*, 1992; Prange and Streeck, 1995].

The 39 or 42 kDa large preS1 protein is the 389 or 400 amino acid translational product of the 2.4 kb mRNA transcript [Seeger, 1994]. Because preS1 translation is initiated from the first of the three S gene start codons, this protein contains three domains – preS1 (108 or 119 aa), preS2 (55 aa), and S (226 aa) – and therefore shares a carboxy-terminal sequence with the middle and small surface proteins (*Figure 1.4 and 1.5a,b*). Present primarily in Dane particles, at levels approximately 5 to 15% of HBsAg, preS1 proteins are exposed on the virion or particle surface [Seeger, 1994], where both the preS2 and S domain of each protein are covered by the preS1 domain [Locarnini *et al.*, 2003]. The large surface protein is fixed to the viral envelope by a myristic acid group that is acylated to the protein's N-terminal glycine residue [Ganem, 1996]. Such myristylation is not required for virion assembly, but is essential for infectivity [Strauss and Strauss, 2002].

In contrast to the other surface proteins, the preS1 protein assumes two different topologies – its preS1 domain can be located either in the ER, and thus ultimately on the virion surface, or in the cytosol, to be localised in the virion interior (*Figures 1.5a and 1.5b*) [Kann, 2002]. The proteins with preS1 domains exposed on the virion surface function as ligands for HBV attachment to hepatocytes, via the prominent cell attachment epitope of preS1 located between amino acids 21 and 47 [Neurath *et al.*, 1986]. There is controversial

evidence that the host cellular receptor mediating this viral attachment and entry is annexin V, a calcium-binding protein that occurs in small amounts on cytoplasmic membranes [Hertogs *et al.*, 1993; Carman, 1996]. In contrast, the preS1 proteins that have preS1 domains located in the hepatocyte cytosol are involved in the envelopment of mature core particles and are therefore essential to virion assembly [Ngui *et al.*, 1999; Kann, 2002]. The nucleocapsids bind directly to a linear stretch of 22 amino acids between residues 103 and 124 within the preS1 proteins, which results in budding of the nucleocapsids through the ER membrane and their concomitant envelopment [Ponsel and Bruss, 2003].

Importantly, the preS1 envelope protein contains a number of highly immunogenic T- and B-cell epitopes that appear to be critical for host protection or recovery from viral infection [Locarnini *et al.*, 2003]. The major immunogenic B-cell epitopes, against which antibodies are formed, occur at amino acids 27 to 35, 72 to 78, and 95 to 107, while the two T-cell epitopes are at residues 21 to 48 and at 81 to 108 [Chisari and Ferrari, 1995].

Translation of the 2.1 kb mRNA transcript produces the 31 or 33 kDa middle preS2 protein containing 281 amino acids [Seeger, 1994]. PreS2 translation is initiated from the second of the three S gene start codons, such that the protein has two domains, preS2 (55 aa) and S (226 aa). Consequently, preS2 shares a carboxy-terminal sequence with the small surface protein (*Figures 1.4 and 1.5c*). Occurring at levels that are 1 to 2% of HBsAg, preS2 proteins are minor components on the surface of HBV virions and subviral particles, where the preS2 domain covers that of S [Kann, 2002; Seeger, 1994]. In addition to glycosylation within the S domain, preS2 is N-glycosylated at amino acid 4 (36 kDa protein) and, in HBV genotypes B to F, it may be additionally O-glycosylated at residue 37 [Kann, 2002].

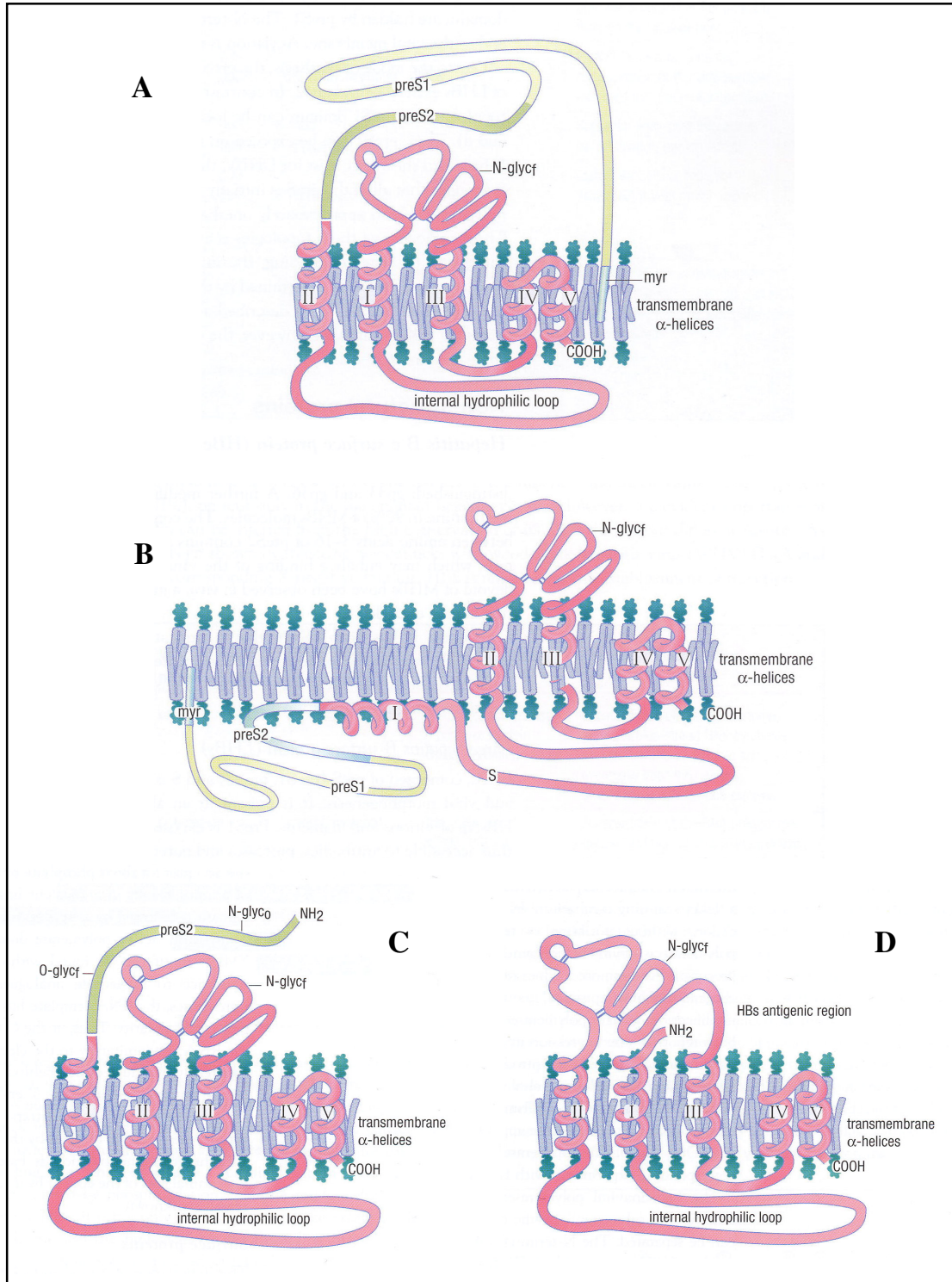


Figure 1.5 Topological models of the three HBV surface proteins. The top of each figure represents the lumen of the ER / surface of the virus, while the bottom corresponds to the cytosol / virus interior. The lipid bilayer is shown in blue. (A) PreS1 protein with ER localised PreS1 domain. (B) Cytoplasmically localised PreS1 domain. (C) PreS2 protein. (D) HBsAg protein [Kann, 2002].

A further modification in 90% of preS2 antigens is the acetylation of the N-terminal methionine residue [Kann, 2002].

The preS2 protein is not essential for HBV replication, virion morphogenesis, or secretion, and does not have a major function in infectivity [Fernholz *et al.*, 1993]. The preS2 domain does, however, display four putative functions. It has a conformation-independent immunogenic region between amino acids 3 and 16 that contains a binding site for polymerised human serum albumin (pHSA) [Yu *et al.*, 1985; Kann, 2002; Shouval, 2003]. Given that hepatocytes also display a receptor for pHSA, it is postulated that this protein may enhance binding of the virus to the cell via preS2 [Tiollais *et al.*, 1985]. Additionally, preS2 has an activated protein kinase binding site and a permeabilisation site, which may be important in penetration of the host cell and transfer of HBV into the cytosol [Shouval, 2003]. PreS2 antigens also express a highly immunogenic T-cell epitope (aa 30-55) and B-cell epitope (aa 14-24) that, together with preS1 antigens, induce neutralising antibodies able to block attachment, endocytosis, and possibly membrane penetration of HBV into the hepatocyte [Feitelson, 1994; Shouval, 2003].

Consisting of only the S domain, HBsAg is the smallest of the three surface proteins, and is the 24 or 27 kDa, 226 amino acid translational product of the 2.1 kb mRNA transcript (*Figure 1.4*). This antigen is the major envelope protein in HBV virions and subviral particles, representing 85% of the total number of surface proteins [Kann, 2002]. As such, HBsAg is the most useful indicator in serum of acute or, if present for longer than six months, of chronic HBV infection [Francois *et al.*, 2001]. Each small surface protein consists of five hydrophobic, membrane-spanning domains and three hydrophilic, extramembranous domains (*Figure 1.5d*) [Berting *et al.*, 1995; Kann, 2002]. Important in

anchoring the HBsAg to the envelope, the hydrophobic domains are folded into α -helices and constitute about 50% of the protein [Berting *et al.*, 1995].

The first hydrophobic helix (I), termed signal I and located between amino acids 11 and 29, crosses the ER membrane and mediates the translocation of the upstream sequence into the ER lumen [Berting *et al.*, 1995; Prange *et al.*, 1995]. Following this, amino acids 30 to 79 constitute the first polar hydrophilic domain, which forms an internal loop within the cytoplasm and is subsequently localised in the virion interior. As such, this domain facilitates the attachment and envelopment of HBV nucleocapsids from the cytosol via a binding site between residues 35 and 46 [Ponsel and Bruss, 2003]. Signal II, the second hydrophobic helix (II), comprises residues 80 to 98 and is able to insert into the ER membrane to mediate the translocation of the downstream sequences [Berting *et al.*, 1995].

Following helix II is the second hydrophilic domain of HBsAg that consists of 70 amino acids spanning residues 99 to 168 (*Figure 1.5d*). Importantly, this large domain is present in the ER lumen and is therefore exposed on the surface of HBV virions and subviral particles. Approximately 40% of HBsAg proteins, having molecular weights of 27 kDa, are N-glycosylated with oligosaccharide chains at asparagine¹⁴⁶ within this domain [Peterson *et al.*, 1982; Berting *et al.*, 1995]. The entire domain contains a high number of cysteine residues that are cross-linked by disulphide bonds, forming a conformational loop structure that constitutes the major antigenic determinant of HBsAg – the *a* determinant [Carman *et al.*, 1990]. The antibodies induced by the neutralising epitopes within this *a* determinant of HBsAg are sufficient to provide protective immunity against HBV infection and aid in its clearance [Hsu, 1997].

Located downstream from the surface-exposed major hydrophilic region of HBsAg (amino acids 169 to 226), helix III traverses the non-polar lipid membrane, followed by a short stretch of hydrophilic, cytosolic amino acids, and two additional transmembranous α -helices (IV and V) (*Figure 1.5d*) [Berting *et al.*, 1995; Kann, 2002; Torresi, 2002]. This carboxy-terminus of HBsAg is important in orientating the protein in the ER membrane for accessibility to the glycosylation machinery [Wang *et al.*, 1996]. In addition to its crucial importance as a major antigen, HBsAg bears the signals necessary for correct assembly of envelope proteins and lipids into virions and subviral particles, and for their subsequent secretion from host cells [Mangold and Streek, 1993]. Finally, there is evidence for a hepatocyte-binding epitope within the S protein that acts synergistically with that of the preS1 protein to mediate effective cell attachment [Paran *et al.*, 2003].

1.1.7 Replication

Unique to animal DNA viruses, the HBV genome is replicated by viral reverse transcriptase. Binding of HBV preS1 and S protein ligands to cell receptors mediates the tissue-specific infection by HBV of a hepatocyte. Following the subsequent viral internalisation and uncoating, the HBV nucleocapsid releases its DNA into the cell nucleus where positive-strand synthesis is completed by endogenous viral DNA polymerase activity, thus converting the relaxed circular DNA genome into a covalently closed circular (cccDNA) molecule [Ganem, 1996].

In the nucleus, transcription of the genome by host RNA polymerase II then yields the four mRNAs already described, the largest of which (3.5 kb), termed pregenomic RNA (pgRNA), is characterised by 200 nt redundant 5' and 3' ends that include 11 nt direct repeats (DR1, at both ends, and DR2, at 3' end) [Seeger, 1994; Ganem, 1996]. In the cytoplasm, some of the pgRNA is translated into core proteins and DNA polymerase (reverse transcriptase), while the other transcripts encode the surface proteins, HBeAg, and HBxAg. The inverted repeat sequences at the 5' end of the remaining untranslated pgRNA subsequently fold into a 70 nt bipartite stem-loop structure called the encapsidation signal or epsilon (ϵ), which binds to the terminal protein domain of polymerase and activates the enzyme using the host molecular chaperones Hsp60 and Hsp90 [Nassal and Schaller, 1993; Kramvis and Kew, 1998; Seeger and Mason, 2000].

Following binding of polymerase, ϵ encapsidates or packages the pgRNA and polymerase into subviral particles composed of HBcAg subunits [Pollack and Ganem, 1993]. The bound activated polymerase subsequently acts as a primer for RNA-dependent negative-strand DNA synthesis (reverse transcription) (*Figure 1.6*) [Wang and Seeger, 1992]. Priming occurs within the six nucleotide bulge of 5' ϵ at the 5' UUC 3' motif, using the hydroxyl group of Tyr⁶³ within the terminal protein domain as a substrate for the formation of a phosphodiester bond with dGMP [Seeger and Mason, 2000]. This reaction leads to the synthesis of the first three nucleotides (5' GAA 3') of negative strand DNA, which are then translocated to the complementary sequence of DR1 at the 3' end of the pgRNA, where DNA synthesis continues to the 5' end of the pregenome [Ganem, 1996; Locarnini *et al.*, 2003].

Occurring concomitantly with negative-strand DNA synthesis, the pgRNA strand is degraded by the RNase H activity of the polymerase, leaving 18 nucleotides (including the DR1 sequence) at the 5' end to prime the subsequent synthesis of positive-strand DNA [Ganem, 1996]. This synthesis occurs following the transfer and annealing of the RNA primer to the complementary DR2 sequence at the 5' end of the DNA strand [Seeger and Mason, 2000]. Positive-strand elongation proceeds to the 5' end of the negative-strand of DNA and includes a short terminal redundancy that is complementary to one occurring at the 3' end of the negative-strand. As such, the 3' end of the growing positive-strand can be transferred from the terminal protein-linked 5' end to the 3' end of the negative-strand, thereby circularising the genome and allowing continuation of positive-strand synthesis for a variable length (*Figure 1.6*) [Ganem, 1996; Locarnini *et al.*, 2003].

The viral nucleocapsid, containing the newly synthesised relaxed circular double-stranded DNA, with a polymerase covalently attached to the 5' end of its negative-strand, either recycles the DNA back to the nucleus for conversion into cccDNA, or it transfers from the cytoplasm to the ER, into which it buds to acquire an envelope containing ER membrane lipids and all three viral surface proteins [Ganem, 1996; Seeger and Mason, 2000]. The enveloped virion is finally transported through the ER into the Golgi complex, where the surface proteins become glycosylated prior to secretion of the mature virion from the cell into the bloodstream via the constitutive secretory pathway, for infection of another hepatocyte [Seeger and Mason, 2000; Locarnini *et al.*, 2003].

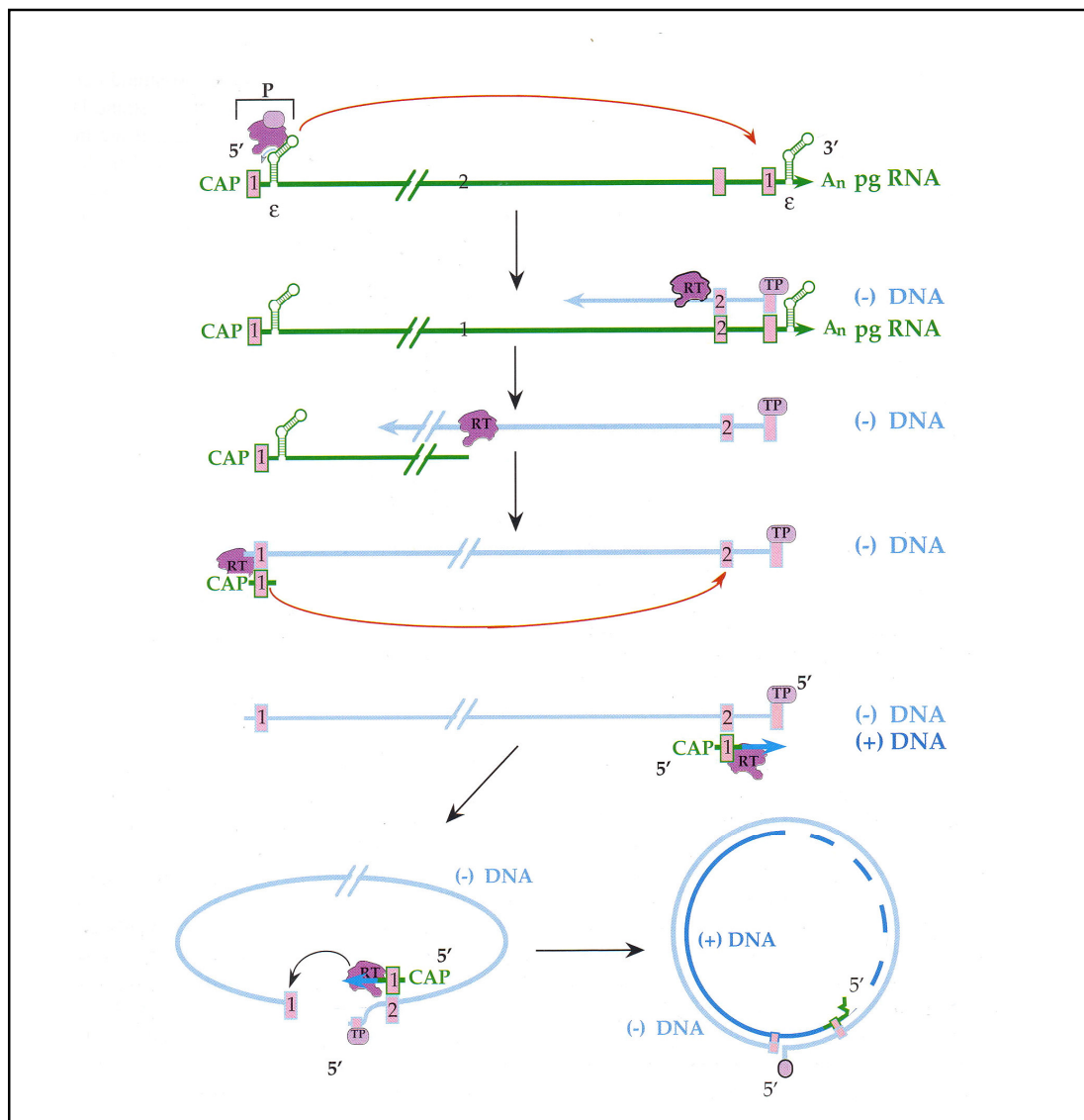


Figure 1.6 HBV DNA synthesis by reverse transcription during replication. pgRNA, pregenomic RNA; RT, reverse transcriptase; TP, terminal protein; 1 and 2 in pink blocks, direct repeats 1 and 2 [Strauss and Strauss, 2002].

1.1.8 Host Immune Responses Targeting HBV

The host mounts a complex of immune responses against invading HBV. Following infection of hepatocytes, an apparent initial quiescent phase of 4 to 7 weeks occurs prior to the vigorous replication of HBV, which reaches levels of 10^9 to 10^{10} virions/ml in 1 to 2 weeks and infects most hepatocytes [Bertoletti and Ferrari, 2003].

In an acute HBV infection, this rapid peak in viral replication is accompanied by the deployment of host innate responses, including phagocytes, the complement system, and natural killer cells, in response to the production of interferon (IFN)- α and IFN- γ by infected hepatocytes [Ferrari *et al.*, 2003]. Seven to ten weeks after infection, these responses subsequently trigger the adaptive immunity, which involves the action of T-helper (CD4+) lymphocytes, cytotoxic T (CD8+) lymphocytes, and antigen presenting cells (APCs) such as macrophages and dendritic cells [Francois *et al.*, 2001; Bertoletti and Ferrari, 2003]. Once CD4+ T-cells recognise the viral nucleocapsid antigens presented by major histocompatibility complex (MHC) class II molecules on the surface of APCs, they proliferate and produce cytokines to stimulate antiviral humoral and cellular responses. Similarly, following their recognition of viral envelope, nucleocapsid and polymerase antigens bound to MHC class I molecules on APCs, or on infected hepatocytes, HBV-specific CD8+ T-cells are stimulated either to lyse the APCs, to lyse the specific hepatocytes, or to release interferon- γ and tumour necrosis factor (TNF)- α , which are able to down-regulate viral replication in surrounding hepatocytes without directly killing the cells [Jung and Pape, 2002; Ganem and Prince, 2004].

Activation of the HBV-specific cellular immunity is followed at 10 to 12 weeks post-infection by the adaptive humoral responses, involving the T-cell dependent production of antibodies by B-lymphocytes [Bertoletti and Ferrari, 2003]. Antibody production is essential for the neutralisation of free HBV antigens and for interference with viral entry into hepatocytes. Three different categories of antibody response are mounted against the viral antigens [Yamamoto *et al.*, 1994]. First, antibodies against the viral core antigen (HBcAg) are raised. Following this, antibodies are raised to HBeAg, and finally to the small surface protein, HBsAg, for termination of the HBV infection [Yamamoto *et al.*, 1994]. The production of the latter two antibodies is strictly dependent on the helper effect of CD4+ T-cells, through both cognate interaction and the release of cytokines necessary for differentiation of B-cells into antibody-producing cells [Ferrari *et al.*, 2003]. Although anti-HBc does not interfere with viral replication, anti-HBe and anti-HBs, which are present in high titres in acutely infected hosts, are crucial in preventing the life cycle of HBV [Yamamoto *et al.*, 1994].

Therefore, for the resolution of an acute infection, anti-HBs forms immune complexes with HBsAg on subviral particles and virions, removing them from circulation and therefore preventing viral attachment to, and uptake by host cells [Francois *et al.*, 2001]. The integrated activation of both the cellular and humoral immune responses is thus necessary for the control of HBV infection [Bertoletti and Ferrari, 2003].

Chronic infections represent failure of the host's normal defence mechanisms to eradicate the virus, because of either an imperfectly functioning cellular and humoral immune system or evasion strategies of HBV such as the formation of mutants [Francois *et al.*, 2001; Ferrari *et al.*, 2003].

1.1.9 HBV Variants

Variants of HBV evolve rapidly because the DNA virus replicates by the reverse transcription of pregenomic RNA, a process susceptible to errors due to the lack of enzymatic proofreading activity [Steinhauer and Holland, 1986; Yamamoto *et al.*, 1994]. Counteracting this, however, is its extremely compact genome that requires sequence conservation and that therefore prevents the occurrence of a large degree of genetic variability [Mizokami *et al.*, 1997].

HBV replication consequently leads to the mutation of 1.4×10^{-5} to 5×10^{-5} bases per site per year [Okamoto *et al.*, 1987; Orito *et al.*, 1989; Mimms, 1995; Fares and Holmes, 2002] – a mutation rate that is approximately the same as retroviruses and 10^4 times greater than that of other DNA viruses. Since HBV infections may persist for many years, these mutations may accumulate and become clinically significant if they do not interfere with viral processes [Francois *et al.*, 2001]. As such, two classes of variants can be discerned. Natural variants possess mutations that may have been selected over centuries of infection or over the course of a single infection, and are dependent on the unique immune system dictated by the genetic background of the host [Carman, 1996]. Occurring more frequently, however, variants selected over a short period by human intervention display mutations induced by the selective pressure from the narrow range of antibodies that are produced following vaccination, hepatitis B immunoglobulin (HBIG) administration, or antiviral therapy with nucleoside analogues such as Lamivudine and, less frequently, adefovir [Carman, 1996].

1.1.9.1 Genotypes

According to comparisons of the complete genome sequences of HBV, natural variation of the virus has resulted in its classification into eight genotypes, A to H, displaying an intergenotypic divergence of more than 8% [Okamoto *et al.*, 1988; Norder *et al.*, 1992b; Naumann *et al.*, 1993; Stuyver *et al.*, 2000; Arauz-Ruiz *et al.*, 2002]. Each genotype has a differing genomic length and shows a distinct geographical distribution, which often reflects the patterns of human migration (*Table 1.1*).

Genotype A is prevalent in Northwest Europe, North America, and sub-Saharan Africa, and includes a unique subgroup that displays a nucleotide divergence of 4.6% from genotype A, termed subgenotype A1, that occurs in South Africa [Norder *et al.*, 1993; Bowyer *et al.*, 1997; Kramvis *et al.*, 2002], Asia, and South America [Kramvis *et al.*, 2005]. Genotypes B and C occur in the indigenous populations of Southeast Asia, Australia and the Pacific islands, where perinatal transmission is the main source of HBV infection, whereas genotype D is distributed worldwide, predominating in the area ranging from Southern Europe and North Africa to India, and in West and South Africa where horizontal transmission is more prevalent (*Table 1.1*) [Okamoto *et al.*, 1988; Norder *et al.*, 1993; Bowyer *et al.*, 1997; Orito *et al.*, 2001]. The most similar to genotype D genetically, genotype E occurs amongst Africans [Norder *et al.*, 1993], while the most divergent of the genotypes, F, is found in South and Central America and in Polynesia [Norder *et al.*, 1993; Arauz-Ruiz *et al.*, 1997]. To date, genotype G has been limited to HBV carriers in France, Germany, Italy, the United Kingdom, and the United States of America [Stuyver *et al.*, 2000; Vieth *et al.*, 2002; Westland *et al.*, 2003], as has the recently identified genotype H to carriers in Central America [Arauz-Ruiz *et al.*, 2002].

Because of the presence of several different genotypes in the same geographical area, recombination between genotypes is possible in a host following simultaneous transmission of several genotypes (co-infection) or from sequential infections with different genotypes (superinfection) [Kidd-Ljunggren *et al.*, 2002]. Recombination between genotypes B and C has been described in Asia, where both genotypes are endemic [Morozov *et al.*, 2000], while in South Africa, recombination occurs between the two prevalent genotypes, A and D [Owiredu *et al.*, 2001].

1.1.9.2 Serological Subtypes

Prior to the identification of genotypic variation, nine HBV serotypes or subtypes were described based on their differentiation by monospecific antisera. The two mutually exclusive subtype determinants, *d* or *y* and *w* or *r*, reside in the HBsAg together with the main antigenic determinant *a*, resulting in subtypes *ayw1* to *ayw4*, *ayr*, *adw2*, *adw4*, *adr* and *adrq* [Le Bouvier, 1971; Bancroft *et al.*, 1972; Couroucé *et al.*, 1976]. It has since been shown that the subtype determinants are specified by a change in one single amino acid, at positions 122 (*d* or *y*) and 160 (*w* or *r*) in the HBsAg protein, respectively [Peterson *et al.*, 1984; Okamoto *et al.*, 1987]. Subtype determinants *d* and *w* therefore have a lysine residue at both positions, whereas an arginine at both positions specifies *y* or *r*. In addition, subtype determinant reactivities have been mapped to amino acid positions 127, 134, 140, 143, and 159, which specify *w1* to *w4*, and to positions 177 and 178 for *q* specificity (Table 1.2) [Norder *et al.*, 1992a].

Table 1.1 Relationship between HBV genotypes and subtypes, and corresponding characteristics*

Genotype	Subtype	Genome Length (nt)	Geographical Distribution	Main Route of Transmission
A	<i>adw2</i> <i>ayw1</i>	3 221	North America Northwest Europe Sub-Saharan Africa	Horizontal
B	<i>adw2</i> <i>ayw1</i>	3 215	China Indonesia Japan Taiwan	Vertical
C	<i>adw2</i> <i>adr</i> <i>adrq</i> <i>ayr</i>	3 215	Australia China Japan Korea Polynesia Vietnam	Vertical
D	<i>ayw2</i> <i>ayw3</i> <i>ayw4</i>	3 182	Australia India Mediterranean Region North America Russia West and South Africa	Horizontal
E	<i>ayw4</i> <i>adw2</i>	3 212	West Africa	Horizontal
F	<i>adw4</i>	3 215	Central and South America	Horizontal
G	<i>adw2</i>	3 248	France Germany USA	Horizontal
H	<i>adw4</i>	3 215	Central America	Horizontal

* Adapted from Bartholomeusz and Schaefer (2004).

The nine HBV subtypic variants show a distinctive geographical distribution [Couroucé-Pauty and Soulier, 1983] but do not necessarily correlate with the eight genotypes, as several genotypes are subtypically heterogeneous (*Table 1.1*) [Norder *et al.*, 1992b]. Subtyping has proven its value in the differentiation of HBV strains in clinical, virological, and epidemiological studies, although it is gradually being replaced by genotyping [Norder *et al.*, 1993].

Table 1.2 Amino acid residues within HBsAg specifying the antigenic subtype determinants of HBV

Amino Acid Position	Amino Acid	Subtype Determinant
122	Lysine	<i>d</i>
	Arginine	<i>y</i>
126	Isoleucine	<i>r</i>
127	Proline	<i>w1 / w2</i>
	Threonine	<i>w3</i>
	Leucine	<i>w4</i>
134	Tyrosine	<i>w1</i>
	Phenylalanine	<i>w2</i>
140	Serine	<i>w4</i>
143	Serine	<i>w1</i>
	Threonine	<i>w2</i>
159	Glycine	<i>w1</i>
	Alanine	<i>w2</i>
160	Lysine	<i>w</i>
	Arginine	<i>r</i>
177	Alanine	<i>q⁻</i>
178	Glutamic Acid	<i>q⁻</i>

1.2 HBV VACCINES

1.2.1 Development

The burden of hepatitis B virus infection on the world population necessitated the development of a hepatitis B vaccine that prevented infection – an achievement made possible only following the discovery of HBsAg by Blumberg and colleagues in 1965. Following initial investigations regarding active immunisation against hepatitis B by Krugman *et al.* (1971), Hilleman *et al.* (1978), and Purcell and Gerin (1978), the first hepatitis B vaccine became available for general use in 1982 [Krugman, 1982]. Not only was it the world's first licensed viral subunit vaccine, but, more importantly, it was the world's first licensed vaccine against a human cancer, HCC [Hilleman, 2003]. Because the dominant neutralising antibodies preventing HBV infection are directed against the HBsAg, these vaccines contained highly purified, formalin- or heat-inactivated, alum-adsorbed empty subviral (22 nm) particles composed of HBsAg that had been derived from the plasma of chronically infected individuals [Hollinger, 1996]. Such plasma-derived vaccines were proven to be highly immunogenic, efficacious, and safe [Szmunn *et al.*, 1980].

However, concerns about the safety of the blood products from which plasma-derived vaccines were manufactured, as well as its inconsistency as a source of raw material, led to the development in 1986 of the world's first recombinant DNA vaccine [McAleer *et al.*, 1984; Emini *et al.*, 1986]. Using recombinant DNA technology, the HBV S gene was cloned, inserted into an expression plasmid, and then introduced into genetically manipulated yeast cells (*Saccharomyces cerevisiae*) or mammalian cells (Chinese hamster

ovary or CHO cells) for expression of HBsAg proteins [Emini *et al.*, 1986]. Alum (aluminium hydroxide), a stabilising adjuvant that is a potent B-cell stimulator, and the preservative thiomersal were also added to the vaccines, which demonstrated excellent efficacy, safety, and immunogenicity [Yeoh *et al.*, 1986; Duclos, 2003]. In addition to being more affordable than the plasma-derived vaccines, recombinant DNA vaccines were preferable as they had a better acceptance and availability, and produced only mild and transient untoward reactions [Francois *et al.*, 2001].

1.2.2 Immune Response

Following HBV vaccination with plasma-derived or recombinant HBsAg, the host immune system is induced to produce antibodies against the HBsAg (anti-HBs) and to proliferate HBsAg-specific cytotoxic and helper T-cells. Such an immune response requires three doses of vaccine delivered by intramuscular injection, and provides long-term protection against HBV infection [Francois *et al.*, 2001; Alter, 2003]. HBV vaccines are capable of providing immunity from infection (anti-HBs $\geq 10\text{mIU/ml}$) in 95% of infants, children, and adults [Shouval, 2003; Lavanchy, 2004].

1.2.3 Implementation of Vaccination

Because of the relatively limited availability and high cost of HBV vaccines in the 1980s, vaccination programmes were initially targeted to persons who were at highest risk of infection, such as infants born to HBsAg-positive mothers, healthcare workers exposed to

blood products, household contacts of HBV carriers, haemodialysis patients, and intravenous drug users [Bonanni *et al.*, 2003]. However, it soon became apparent that the vaccine would be most beneficial in its administration to infants worldwide, especially to those born to HBeAg-positive mothers [Hilleman, 2003].

Therefore, in 1992, with an increase in the availability of safe and effective HBV vaccines, the World Health Assembly recommended the addition of HBV vaccination to all national immunisation programmes [World Health Organisation, 1992]. As a consequence, in the 20 years since the development of HBV vaccines, 181 countries have implemented routine infant vaccinations and over one billion people have been immunised worldwide [Namgyal, 2003; Shouval, 2003]. Such universal vaccination not only reduces the number of new HBV infections, but also reduces the burden of HBV-related liver diseases such as cirrhosis and HCC.

1.2.4 Vaccination Programme in South Africa

Of the approximately 387 million carriers of HBV worldwide, four million reside in South Africa, an area in which HBV infection is hyperendemic amongst the black population [Tsebe *et al.*, 2001]. Because the predominant mode of HBV transmission in South Africa, the same as in the rest of sub-Saharan Africa, is the horizontal route, with most children already infected by the age of five years, the only logical approach to the control of HBV infection in this region is to break the cycle of transmission in early childhood [Kew, 1996; Tsebe *et al.*, 2001]. Because active immunisation by vaccination has been proven to be the

most effective way of controlling hepatitis B, the World Health Organisation (WHO) recommended that all countries with moderate or high HBV endemicity institute routine immunisation of all infants by 1995. Accordingly, the South African government introduced the HBV vaccine into its existing Expanded Programme of Immunisation (EPI) in April 1995 [Department of Health, 1995].

Initially (1995-1998), immunisations were performed using the plasma-derived Hepaccine-B vaccine (Cheil Foods and Chemicals Company, Seoul, Korea), which was replaced in 1999 by the recombinant yeast-derived vaccine, Engerix-B (SmithKline Beecham, Philadelphia, USA) [Tsebe *et al.*, 2001]. Both vaccines are administered to children at 6, 10, and 14 weeks, together with the oral polio vaccine, diphtheria-tetanus-pertussis, and *Haemophilus influenzae* type b vaccines [Tsebe *et al.*, 2001]. The HBV vaccine has already shown a positive impact in the elimination of the HBsAg carrier rate in South African children younger than five years [Tsebe *et al.*, 2001], which has consequently decreased the transmission rate of the virus, resulting in a significant degree of protection for both immunised and non-immunised individuals [Schoub *et al.*, 2002].

1.3 SURFACE GENE MUTATIONS AND VACCINE ESCAPE

Despite the great success of the hepatitis B vaccine in preventing infections, the immunological pressure exerted on HBV by universal vaccination has resulted in the selection of various mutants of the virus that are able to escape neutralisation by vaccine-induced antibodies (anti-HBs). Such vaccine escape mutants pose a risk to the community,

because current hepatitis B vaccines and HBIG are not effective in preventing infection with them [Yamamoto *et al.*, 1994]. Moreover, blood units infected with these mutants would be missed by routine screening for HBsAg, resulting in transmission of HBV to recipients [Yamamoto *et al.*, 1994]. However, it has not been shown that the emergence of such mutants has threatened the success of the global vaccination strategy.

HBV immune escape mutants display mutations within the section of the S gene that encodes the *a* determinant of the HBsAg. Based on the model of HBsAg proposed by Stirk *et al.* (1992), the *a* determinant is located within the extra-membranous major hydrophilic region (MHR) that spans amino acids 99 to 168. Traditionally considered to form a two-loop structure of 28 residues between amino acids 124 and 147 [Ashton-Rickardt and Murray, 1989], the *a* determinant is now thought to form a more extensive ‘cysteine web’ structure consisting of two loops between aa 107 and 138, and between aa 139 and 147 or 149 [Chen *et al.*, 1996]. In addition, a third, tight loop occurs from aa 121 to 124 within the first loop [Qiu *et al.*, 1996]. The tertiary structure of the entire *a* determinant is maintained by disulphide bridges between its eight cysteine residues (*Figure 1.7*), resulting in a highly conformational structure upon which its antigenicity is dependent [Imai *et al.*, 1974].

The *a* determinant contains the immunodominant B-cell epitopes against which neutralising antibodies (anti-HBs) are raised. Phage display libraries have revealed the presence of three continuous epitopes between codons 115 and 129 [Motti *et al.*, 1994], 117 and 122, and 121 and 124 [Germaschewski and Murray, 1996], as well as several discontinuous epitopes between aa 101 and 207 [Chen *et al.*, 1996]. Most anti-HBs occurring in sera of vaccinees binds to the epitopes located between amino acids 124 and

147 [Howard *et al.*, 1984], although the second loop of the α determinant containing aa 139 to 147 represents the major neutralising epitope [Steward *et al.*, 1993].

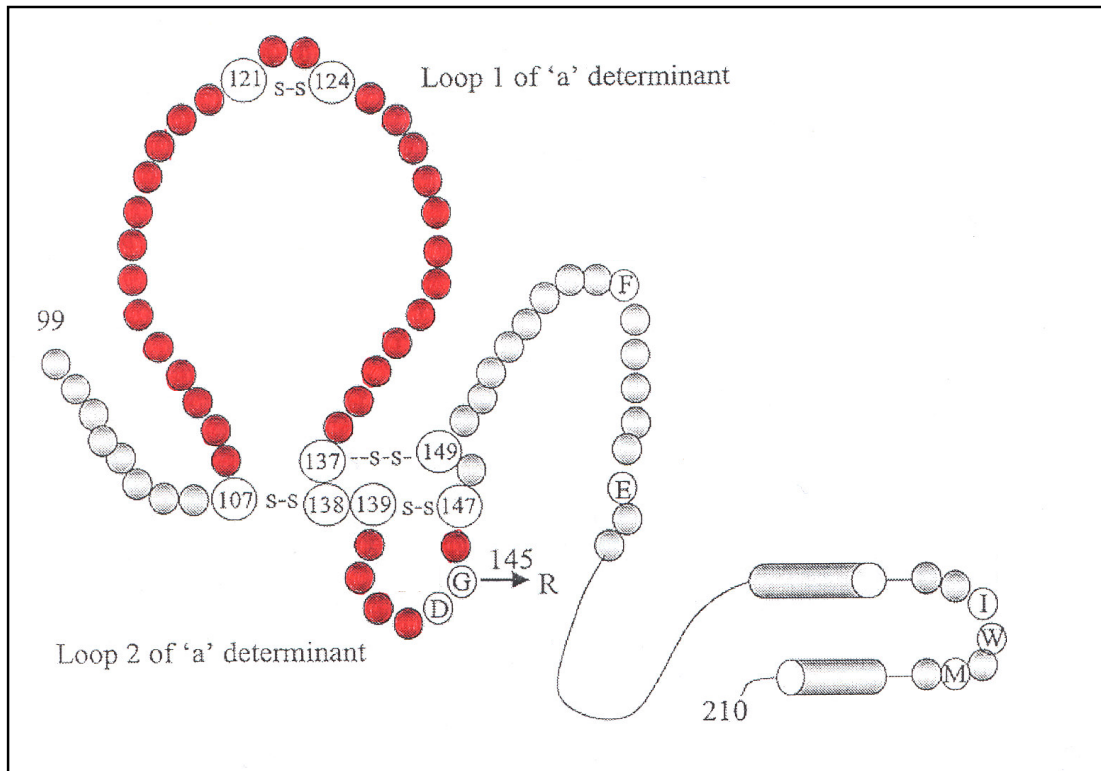


Figure 1.7 Diagrammatic representation of the major hydrophilic region of the HBsAg (aa 99-168) occurring on the surface of the viral envelope. The conformation of the two major loops of the α determinant (red circles) is dependent on the presence of several disulphide bridges (S-S) between its eight cysteine residues (large circles). The most common vaccine escape mutation, G145R, is shown within the second loop. The carboxyl end of the protein, containing two alpha-helices (cylinders), is embedded in the viral envelope [Adapted from Torresi *et al.*, 2002].

Mutations occurring within the α determinant, especially between aa 139 and 147, produced as a result of errors incurred during reverse transcription (replication), have the potential to disrupt the tertiary structure of the epitopes and hence decrease their antigenicity. Not only does this decrease or prevent antibody formation against the mutant

HBV, it also prevents recognition of, and binding to, the HBsAg epitopes by vaccine-induced antibodies, thereby allowing the virus to escape neutralisation (vaccine escape). Such mutant viruses consequently become the dominant viral species in a host as a result of the selection pressure of vaccination.

The first HBV vaccine escape mutant to be described occurred in an infant born to a chronic HBV carrier mother in Italy [Carman *et al.*, 1990]. Despite receiving both active and passive immunisation at birth and subsequently developing an adequate anti-HBs response, the child became chronically infected with an HBV mutant displaying a single amino acid substitution of glycine to arginine at residue 145 (G145R), caused by a guanine to adenine point mutation at nucleotide 587 in the S gene [Carman *et al.*, 1990]. This mutant has since been described in vaccinated children born to HBeAg-positive carrier mothers around the world [Fujii *et al.*, 1992; Okamoto *et al.*, 1992; Oon *et al.*, 1995; Nainan *et al.*, 1997; Lee *et al.*, 2001], in other vaccinees, and in liver transplant recipients receiving monoclonal anti-HBs therapy [McMahon *et al.*, 1992], and is thus the prototype and most common escape mutant. It is viable, infectious, pathogenic [Ogata *et al.*, 1997], and stable [Oon and Chen, 1998]. Various other escape mutations within the *a* determinant have been reported from around the world, particularly from the East (*Table 1.3*). Such viruses are most prevalent in countries with high endemic rates of HBV infection and with established HBV vaccination programmes [Hsu *et al.*, 1999].

In addition to occurring in vaccinees, in whom selection is provided by the narrow range of vaccine-induced antibodies, immune escape mutants occur in chronic HBV carriers with [Brind *et al.*, 1997] or without immunosuppression [Moriyama *et al.*, 1991]; in patients receiving monoclonal [McMahon *et al.*, 1992] or polyclonal immunotherapy [Carman *et*

al., 1996]; in those infected with HBV that is serologically non-reactive to HBsAg diagnostic assays [Carman *et al.*, 1995]; and, less frequently, in natural isolates [Yamamoto *et al.*, 1994]. Escape mutants therefore pose a potential threat both to the success of vaccination programmes and to the supply of safe blood products in endemic areas of the world.

Table 1.3 Common vaccine-escape mutations found between amino acids 124 and 147 within the *a* determinant of HBsAg.

Wild-Type Amino Acid	Amino Acid Number	Mutant Amino Acid	Country*	Reference
Ile / Thr	126	Ala	Taiwan	Hsu <i>et al.</i> , 1997
		Ser	Japan	Miyake <i>et al.</i> , 1996
Pro / Leu	127	Ser	Italy	Mele <i>et al.</i> , 2001
Gln	129	Arg	Taiwan	Hsu <i>et al.</i> , 1997
		Leu	China	He <i>et al.</i> , 1998
		His	Singapore	Chen <i>et al.</i> , 2002
Gly	130	Asn	Singapore	Chen <i>et al.</i> , 2002
Thr	131	Asn	Canada	Yoshida <i>et al.</i> , 2000
Met	133	Ile	Taiwan	Lee <i>et al.</i> , 1997
		Thr	Canada	Yoshida <i>et al.</i> , 2000
		Leu	Singapore	Chen <i>et al.</i> , 2002
		Val	China	Chen <i>et al.</i> , 2003
Phe	134	Tyr	UK	Ngui <i>et al.</i> , 1997
Cys	137	Arg	Czech Republic	Roznovsky <i>et al.</i> , 2000
Cys	138	Arg	Germany	Sterneck <i>et al.</i> , 1998
Cys	139	Ser	Czech Republic	Roznovsky <i>et al.</i> , 2000
Thr	140	Ser	Japan	Miyake <i>et al.</i> , 1996
Lys	141	Glu	The Gambia	Karthigesu <i>et al.</i> , 1994
		Arg	Singapore	Chen <i>et al.</i> , 2002
Pro	142	Ser	Hong Kong	Seddigh-Tonekaboni <i>et al.</i> , 2001
			Singapore	Chen <i>et al.</i> , 2002
Asp	144	Ala	China	Ni <i>et al.</i> , 1995
			Taiwan	Lee <i>et al.</i> , 1997
			UK	Ngui <i>et al.</i> , 1997
			Singapore	Chen <i>et al.</i> , 2002
Gly	145	Arg	Italy	Carman <i>et al.</i> , 1990
			Singapore	Harrison <i>et al.</i> , 1991
			Japan	Fujii <i>et al.</i> , 1992
			Taiwan	Hsu <i>et al.</i> , 1997
			USA	Nainan <i>et al.</i> , 1997
			Korea	Lee <i>et al.</i> , 2001
			Hong Kong	Seddigh-Tonekaboni <i>et al.</i> , 2001
		Ala	Hong Kong	Seddigh-Tonekaboni <i>et al.</i> , 2001

* Country in which HBV mutant caused vaccine-escape.

1.4 RATIONALE AND AIMS OF THE STUDY

Following the introduction of vaccination against HBV in 1995, there have been no documented vaccine escape mutants of the virus in South Africa. Recently, however, a case of breakthrough HBV infection has occurred in this country: a medical practitioner became HBsAg-positive following a needle-stick injury despite having been previously vaccinated.

The aim of this study was therefore to determine whether this infection was the result of mutations within the S gene, and hence the HBsAg, of HBV that permitted the virus to escape neutralisation by the vaccine-induced anti-HBs. Such information could aid in tracing the molecular evolution of the virus since the introduction of vaccination in this country, and would provide a comparison of South African HBV vaccine-escape mutants with those found in different geographical regions of the world.

Chapter 2: MATERIALS AND METHODS

2.1 SUBJECT AND SAMPLES

The subject of the study was a 55 year old South African Caucasian male who was a medical practitioner in Botswana and who had been immunised in the late 1980s by the intradermal route. Because of doubts concerning the efficacy of intradermal administration of the vaccine, the practitioner gave himself a booster dose by the intramuscular route in 1993. He subsequently suffered a needle-stick injury in June 1999. Blood was taken to determine his anti-HBs titre on the day of the needle-stick injury and he gave himself a further booster of the vaccine. The blood was reported to have an anti-HBs titre of 17.82 mIU/ml. He showed no clinical evidence of HBV infection after the injury, but in January 2002 a routine blood test revealed a serum alanine aminotransferase level of 505 mIU/ml and an aspartate aminotransferase level of 285 mIU/ml. Serological tests were then performed and showed the presence of HBsAg and IgM anti-HBc (anti-HBe was not tested). These raised levels persisted for more than six months, during which time IgM anti-HBc converted to IgG anti-HBc. He was then given a course of Lamivudine, which resulted in the disappearance of HBsAg and normalisation of the serum aminotransferase levels.

Serum samples obtained from the patient were stored at -70°C until analysed. HBV markers were detected in the sera using commercially available kits (Abbott Laboratories, Chicago, IL).

A liver tissue sample was obtained from the patient by biopsy and preserved in paraffin-wax until analysis.

The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (clearance number R14/49 Crowther) and informed consent was obtained from the patient (Appendix B).

2.2 DNA EXTRACTIONS

The hepatitis B viral DNA used in the study was extracted from the serum and liver tissue samples of the patient.

2.2.1 DNA Extraction From Serum

HBV DNA was extracted from the serum samples using the QIAamp Blood Mini Kit (QIAGEN, GmbH, Hilden, Germany), a method for nucleic acid purification and removal of amplification inhibitors. The protocol specified by the manufacturers was followed.

A 200 μ l aliquot of serum was added to 20 μ l QIAGEN Protease (Proteinase K) and 200 μ l Buffer AL in a sterile 1.5 ml microcentrifuge tube and mixed by pulse-vortexing for 15 seconds. The sample was then incubated at 56°C for 10 minutes in order to release a maximum yield of DNA from the degraded virions. Following a brief centrifugation of the

microcentrifuge tube, 200 μ l 100% ethanol was added to the sample and mixed by pulse-vortexing.

The mixture was applied to a QIAamp spin column in a 2 ml collection tube and centrifuged at 8 000 rpm for 1 minute to enable the adsorption of the DNA onto the silica-gel membrane of the spin column. The tube containing the filtrate was replaced with a clean 2 ml collection tube, and the QIAamp spin column was subsequently washed with 500 μ l Buffer AW1 and centrifuged at 8 000 rpm for 1 minute. This latter step was repeated using 500 μ l Buffer AW2 and centrifugation at 14 000 rpm for 3 minutes in order to remove any residual contaminants. Since residual Buffer AW2 in the eluate may cause problems in downstream applications, the spin column was centrifuged again at full speed (14 000 rpm) for 1 minute in a clean 2 ml collection tube. Finally, the purified DNA was eluted from the QIAamp spin column into a sterile 1.5 ml microcentrifuge tube by the addition of 150 μ l preheated (56°C) best quality water, incubation at room temperature (15-25°C) for 5 minutes, and centrifugation at 8 000 rpm for 1 minute. The eluted HBV DNA was stored at -20°C. Known HBV-positive serum and best quality water were used as positive and negative controls, respectively, for the extraction procedure.

2.2.2 DNA Extraction From Liver Tissue

HBV DNA was extracted from the paraffin-wax-embedded liver tissue sample using the DNeasy Tissue Kit (QIAGEN, GmbH, Hilden, Germany), for removal of the tissue from the paraffin-wax, followed by use of the QIAamp DNA Micro Kit (QIAGEN, GmbH,

Hilden, Germany), for purification of total DNA from a small amount of tissue. The protocols followed were those specified by the manufacturers.

A 1 200 μ l aliquot of xylene was added to a small section (less than 25 mg) of paraffin-wax-embedded liver tissue in a 2 ml microcentrifuge tube, vortexed vigorously, and centrifuged at 14 000 rpm for 5 minutes. The supernatant of xylene and dissolved paraffin was removed by pipetting, leaving the tissue as a pellet. To remove residual xylene, 1 200 μ l absolute ethanol was added to the pellet, mixed gently by vortexing, and centrifuged at 14 000 rpm for 5 minutes. The ethanol was removed by pipetting and the step involving addition of ethanol, vortexing, and centrifugation was repeated once. Following removal of the ethanol supernatant by pipetting, the open microcentrifuge tube was incubated at 37°C for 15 minutes until the residual ethanol had evaporated. The resultant tissue pellet was finally resuspended in 180 μ l Buffer ATL.

Using the QIAamp DNA Micro Kit, 20 μ l Proteinase K was subsequently added to the resuspended tissue, mixed by pulse-vortexing for 15 seconds, and incubated at 56°C overnight until the tissue sample had completely lysed under the highly denaturing conditions. A 200 μ l aliquot of Buffer AL was then added to the sample and mixed by pulse-vortexing for 15 seconds, followed by addition of 200 μ l 100% ethanol, pulse-vortexing for 15 seconds, and incubation for 5 minutes. The entire lysate was transferred to a QIAamp MinElute Column and centrifuged at 8 000 rpm for 1 minute, causing the DNA to be adsorbed onto the silica-gel membrane of the column and the proteins and other contaminants to be eluted as filtrate. The tube containing the filtrate was replaced with a clean 2 ml collection tube and, in order to wash away residual contaminants, the QIAamp MinElute Column was subsequently washed with 500 μ l Buffer AW1 and centrifuged at

8 000 rpm for 1 minute. This step was repeated using 500 μ l Buffer AW2 and the 2 ml collection tube was replaced with a clean one. Because ethanol carryover into the eluate may interfere with some downstream applications, the column and collection tube were centrifuged at 14 000 rpm for 3 minutes to completely dry the membrane. Following the placement of the QIAamp MinElute Column in a sterile 1.5 ml microcentrifuge tube, 50 μ l best quality water was added to the centre of the membrane, incubated at room temperature (15-25°C) for 5 minutes, and centrifuged at 14 000 rpm for 1 minute. The HBV DNA was thereby eluted in the distilled water and subsequently stored at -20°C.

2.3 AMPLIFICATION OF HBV DNA

Polymerase Chain Reaction (PCR) is a powerful *in vitro* technique used for enzymatically amplifying small quantities of particular DNA sequences over a billion-fold. Oligonucleotide primers complementary to the regions flanking the 3' ends of both strands of the target sequence are annealed to the DNA template following its denaturation into single strands. Primer extension is then catalysed by thermostable *Taq* DNA polymerase, resulting in duplication of the target sequence. Repetition of these three steps – denaturation, annealing, and extension – via numerous cycles in an automated thermal cycler therefore results in the exponential amplification of the target DNA sequence.

2.3.1 Full-Length Genome Amplification

A single-round PCR using primers corresponding to overlapping sequences within the nick region of the HBV genome [Gunther *et al.*, 1995] was performed in order to amplify the complete, or full-length, genome of the HBV DNA extracted from the serum (*Table 2.1*)

Table 2.1 Oligonucleotide primers used for the PCR amplification of the full-length HBV genome

Primer	Sequence	Position* (nt)	Size† (bp)
P1 (+)	5' TTTTTCACCTCTGCCTAATCA 3'	1821-1841	3215
P2 (-)	5' AAAAAGTTGCATGGTGCTGG 3'	1825-1806	

Abbreviations: (+), sense; (-), antisense

* Nucleotide position of HBV *adv* genome (GenBank accession number AY233276), where position 1 is the *EcoRI* cleavage site.

† Size of the PCR amplicon in base pairs.

The amplification reaction was performed as a 'hot start' PCR and, as such, was prepared as two separate PCR mixes. The reaction mix had a final volume of 20 μ l and consisted of 2 μ l 1X Expand High Fidelity buffer (including 1.5mM MgCl₂); 0.05 μ l, 50 μ M of each of the four dNTPs; 1.25 μ l, 1 μ M of each of the primers; 5 μ l (20% vol/vol) DNA; and 10.3 μ l best quality water. The enzyme mix consisted of 0.75 μ l, 0.1 U/ μ l Expand High Fidelity Enzyme mix (Roche Applied Science, Germany) – containing thermostable *Taq* DNA polymerase and *Tgo* DNA polymerase, a thermostable enzyme with 3'-5' exonuclease proofreading activity; 0.5 μ l 1X Expand High Fidelity buffer; and 3.75 μ l

best quality water, making up a 5 μ l final volume. The concentrations stated were those of the reagents in the total 25 μ l PCR mix.

The ‘hot start’ PCR was performed in a Robocycler[®] Gradient 40 Temperature Cycler (Stratagene, La Jolla, CA). The 20 μ l reaction mix was preheated to 94°C for 160 seconds and the 5 μ l enzyme mix was added at the first annealing step. This was followed by 40 cycles of amplification with a cycle profile of 94°C for 40 seconds (denaturation), 58°C for 90 seconds (annealing), and 70°C for 3 minutes with an increment of 2 minutes after every 10 cycles (extension).

Best quality water, instead of DNA, and HBV-positive controls were included in all PCR reactions to validate amplification results. In addition, to avoid contamination and false-positive results, DNA extraction and PCR amplification were performed in physically separate venues, as was the addition of DNA to the final PCR reaction mixtures.

2.3.2 Surface Gene Amplification

Because of the method of preservation of the liver tissue in paraffin-wax and the nature of the DNA extraction procedure, the length of the extracted HBV DNA was less than 650 bp. As such, the full-length genome PCR could not be performed on this DNA. Instead, nested PCR using primers specific to the surface gene of the HBV genome was performed in order to amplify only this region of the liver-extracted DNA (*Table 2.2*). The same PCR was additionally performed on the serum-extracted DNA samples that had not been

amplified by full-length PCR. Nested PCR involves amplification via two consecutive rounds of PCR, in which second round primers are specific to a region within the first round amplicons, and was necessary in this study due to low amounts of DNA in the samples.

Table 2.2 Oligonucleotide primers used for the PCR amplification of the surface gene of HBV

Primer	Sequence	Position [*] (nt)	Size [†] (nt)
<i>Outer Primers</i>			
230F (+)	5' TCACAATACCGCAGAGTCT 3'	230-249	572
800R (−)	5' AACAGCGGTATAAAGGGACT 3'	801-782	
<i>Inner Primers</i>			
P7 (+)	5' GTGGTGGACTTCTCTCAATTTTC 3'	256-278	541
P8 (−)	5' CGGTATAAAGGGACTCACGAT 3'	796-776	

Abbreviations: (+), sense; (–), antisense

^{*} Nucleotide position of HBV *adw* genome (GenBank accession number AY233276), where position 1 is the *EcoRI* cleavage site.

[†] Size of the PCR amplicon in base pairs.

The final reaction mix for the first round of PCR had a volume of 25 μl and contained 0.1 μl , 0.014 U/ μl Expand High Fidelity Enzyme (Roche Applied Science, Germany); 2.5 μl 1X Expand High Fidelity buffer (including 1.5 mM MgCl_2); 0.5 μl , 200 μM of each of the four dNTPs; 1.25 μl , 1 μM of each of the outer primers; 2.5 μl (10% vol/vol) DNA; and 15.4 μl best quality water. The concentrations stated are those of the reagents in the final 25 μl volume. PCR was performed in an Eppendorf Mastercycler[®] Gradient programmable

thermal cycler (Eppendorf, Hamburg, Germany) using a cycle profile of 94°C for 1 minute (denaturation), 65°C for 5 minutes (annealing), and 72°C for 3 minutes (extension), for 40 cycles, followed by a final extension at 72°C for 10 minutes.

Using the set of inner primers and the DNA produced following first round amplification, the second round of PCR had a 50 μ l reaction mix containing reagents with identical final concentrations, but double the volumes, to those used in the first round. The cycle profile included an initial activation step at 94°C for 3 minutes, followed by 40 cycles of 94°C for 45 seconds (denaturation), 53°C for 1 minute (annealing), and 72°C for 90 seconds (extension), and then 72°C for 7 minutes as a final extension step.

Best quality water, instead of DNA, and HBV-positive controls were included in all PCR reactions to validate amplification results. The same precautions followed during the full-length PCR were strictly adhered to during the nested PCR in order to avoid cross-contamination.

2.4 DETECTION OF AMPLIFIED DNA PRODUCTS

A 5 μ l aliquot of the full-length genome PCR product or the second round nested PCR product was electrophoresed with 7.5 μ l bromophenol blue loading dye adjacent to a 1 kb or 100 bp DNA ladder (Promega, Madison, WI), respectively, on an ethidium bromide-stained 1% agarose gel at ~100 V for 1.5 hours. To avoid contamination, electrophoresis

was performed at a different venue to those used in the previous methods. Bands of the appropriate size (3.2 kb or 541 bp, respectively) were subsequently visualised on the gel under ultraviolet light.

2.5 CLONING OF AMPLIFIED DNA PRODUCTS

Cloning – the insertion of amplified DNA products into plasmid vectors that are subsequently transformed into competent bacterial cells for replication – provides an unlimited supply of the relevant DNA for experimental use and enables the isolation of minor species from mixed populations.

2.5.1 Addition of 3' A Overhangs

Use of the Expand High Fidelity PCR System (Roche Applied Science, Germany) generates a mixture of PCR products possessing either blunt ends or 3' single deoxyadenosine (A) overhangs. Because the linearised plasmid vector used in cloning has overhanging 3' deoxythymidine (T) residues, it is necessary for all of the PCR products to have 3' A overhangs for efficient ligation with the vector.

Therefore, immediately following amplification of the 3.2 kb full-length genome and the 541 bp S gene fragment, each volume of PCR product was treated with a tenth of the volume of TaKaRa Ex *Taq* DNA polymerase (TaKaRa Bio Inc., Japan), an enzyme with a nontemplate-dependent terminal transferase activity that adds a single A to the 3' ends of dsDNA. The mixtures were then incubated at 72°C for 10 minutes for optimal enzyme activity.

2.5.2 DNA Purification

The dsDNA was highly concentrated and purified from enzymes, primers, nucleotides, and salts prior to cloning using the MinElute Reaction Cleanup Kit (QIAGEN, GmbH, Hilden, Germany). The protocol specified by the manufacturers was followed.

A 300 μ l aliquot of binding Buffer ERC was added to each volume of enzyme-treated PCR products, mixed, and applied to a MinElute column in a 2 ml collection tube. In order to bind the DNA to the silica-gel membrane of the column whilst eluting the enzymes, primers, nucleotides, and salts, the spin column was centrifuged at 14 000 rpm for 1 minute. After the flow-through had been discarded and the MinElute column placed back into the same tube, the membrane was washed to remove residual salts by the addition of 750 μ l Buffer PE and centrifugation at 14 000 rpm for 1 minute. The flow-through was discarded and the MinElute column placed back in the same tube, followed by an additional centrifugation at 14 000 rpm for 1 minute to remove residual Buffer PE. The MinElute column was placed in a clean 1.5 ml microcentrifuge tube and, to elute the

purified DNA, 15 μ l best quality water was added to the centre of the membrane, left to stand for 5 minutes, and finally centrifuged at 14 000 rpm for 1 minute. The column was discarded and the DNA was kept on ice until further use.

2.5.3 Cloning and Transformation

Cloning of the amplicons was performed using the TOPO[®] XL PCR Cloning Kit (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer.

In order to increase cloning efficiency, it is preferential to gel-purify long PCR products by separating them from all shorter amplicons. Since the UV light used in visualising traditional ethidium bromide agarose gels may damage the DNA and hence decrease cloning efficiency, crystal violet agarose gels are used for DNA gel-purification.

The volume of the concentrated DNA was increased to 40 μ l by the addition of best quality water. The 40 μ l aliquot of purified full-length genome or second round S gene PCR product was then electrophoresed with 8 μ l of 6X Crystal Violet Loading Buffer adjacent to a 1 kb or 100 bp DNA ladder (Promega, Madison, WI), respectively, on a crystal violet-stained 0.8% agarose gel at 80 V for 2-3 hours. Electrophoresis was performed at a different venue to those used in the previous methods. Bands of the appropriate size (3.2 kb or 541 bp, respectively) were subsequently visualised on the gel under the light of a clean fluorescent light box.

Using a sterile razor blade, the desired band of DNA was excised from the gel, cut into small pieces, and placed in a sterile 1.5 ml microcentrifuge tube. The volume of the agarose pieces was estimated by assuming that 1 mg $\sim$ 1 μ l. A volume of 6.6 M sodium iodide that was 2.5 times the volume of the agarose pieces was added to the tube, mixed vigorously by vortexing, and incubated at 50°C until the agarose had completely melted. Once at room temperature, 1.5 volumes of Binding Buffer was added and mixed well. The entire mixture was subsequently loaded onto a S.N.A.P. purification column in a collection vial and centrifuged at 4 000 rpm for 30 seconds. The flow-through was poured back onto the column and the centrifugation step was repeated. The latter steps were repeated a third time to bind all of the DNA to the column, and the liquid in the collection tube was discarded. The column was then washed by the addition of 400 μ l 1X Final Wash, centrifugation at 4 000 rpm for 30 seconds, and one repetition of these steps. Once the liquid in the collection tube had been discarded after the final centrifugation, the column was centrifuged at 14 000 rpm for 1 minute to dry the column resin. The S.N.A.P. column was transferred to a sterile 1.5 ml microcentrifuge tube and 40 μ l TE Buffer was added to the column resin. After a 1 minute incubation at room temperature that allowed absorption of the buffer into the resin, the column was centrifuged at 14 000 rpm for 1 minute to elute the DNA into the microcentrifuge tube. The column was finally discarded and the purified PCR product was kept on ice until needed.

Each TOPO[®] Cloning reaction was set up immediately following DNA purification. A 1 μ l aliquot of the pCR[®]-XL-TOPO[®] vector (a linearised 3.5 kb DNA plasmid containing a *lac* promoter region, *lacZ α* ORF, multiple cloning site, TOPO[®] cloning site, fusion joint, *ccdB* lethal gene ORF, kanamycin resistance ORF, zeocin resistance ORF, and pUC origin) was added to 4 μ l of the gel-purified PCR product in a sterile 0.2 ml

microcentrifuge tube, mixed gently, and incubated for 5 minutes at room temperature. Immediately after incubation, 1 μ l 6X TOPO[®] Cloning Stop Solution was added to the reaction and mixed for several seconds to prevent further ligation of the PCR product and vector. The tube was briefly centrifuged and then placed on ice.

Transformation was achieved by the addition of 2 μ l of the TOPO[®] Cloning reaction into a vial of 50 μ l One Shot[®] TOP10 chemically competent *E. coli* cells that had been thawed on ice. After mixing very gently, the mixture was incubated on ice for 30 minutes. To enable the transfer of vectors into the cells, the latter were heat-shocked for 30 seconds at 42°C without shaking, and then the tube was immediately placed on ice for 2 minutes. A 250 μ l aliquot of room temperature SOC medium was subsequently added to the tube, which was shaken horizontally at 150 rpm at 37°C for 1 hour to promote growth of the bacterial cells. Finally, using a sterile glass spreader, 150 μ l of the transformation reaction was spread on each of two Luria-Bertani (LB) agar plates, containing 50 μ g/ml of the antibiotic kanamycin, that had been prewarmed to 37°C for 30 minutes. The plates were then inverted and incubated at 37°C overnight to permit bacterial colony growth.

The pCR[®]-XL-TOPO[®] vector used during cloning allows direct selection of recombinant bacteria via disruption of the lethal *E. coli* gene, *ccdB*. Within the vector, the *ccdB* gene is fused to the C-terminus of the *lacZ α* gene. However, ligation of a PCR product with the linearised vector disrupts the expression of the lethal gene fusion, thereby permitting growth of only positive recombinants upon transformation. Additionally, the presence of a kanamycin-resistance gene in the vector allows only transformed bacterial cells to grow on LB-kanamycin plates.

Therefore, following the overnight incubation, all colonies contained recombinants possessing cloned HBV DNA. In order to provide a greater stock of bacteria from each colony prior to inoculation, 50 colonies at a time were individually replated by streaking on new LB-kanamycin agar plates using sterile inoculation loops, and incubated overnight at 37°C. Plates were then sealed and stored at 4°C until further use.

2.5.4 Isolation of Recombinant Plasmid DNA

Prior to the isolation and purification of plasmid DNA from the bacteria for genotyping and sequencing analysis, several progeny colonies grown from each replated initial colony were inoculated, using a sterile inoculation loop, into a sterile 10 ml tube containing 5 ml LB medium and 5 μ l kanamycin. All of the culture tubes, including a negative control containing only LB and kanamycin, were shaken horizontally at 150 rpm at 37°C overnight, in order to enhance reproduction of the transformed bacteria and hence increase the amount of plasmid DNA. Following incubation, the bacterial cells were pelleted by centrifugation of the tubes at 5 000 rpm for 15 minutes at 4°C. The supernatant was subsequently discarded.

The GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO) was used to isolate and purify the plasmid DNA, containing the HBV DNA, from the pelleted bacterial cells. The protocol specified by the manufacturers was followed.

A 200 μ l aliquot of Resuspension Solution was added to each bacterial pellet and pipetted up and down until the mixture was homogenous. The completely resuspended cells were subsequently transferred to a sterile 1.5 ml microcentrifuge tube. In order to lyse the cells, 200 μ l Lysis Solution was added and the tube was immediately gently inverted several times until the mixture became clear and viscous. Within 5 minutes of the lysis reaction, 350 μ l Neutralisation Solution was added and mixed by gentle inversion of the tube, followed by centrifugation at 14 000 rpm for 10 minutes in order to precipitate the cell debris, proteins, lipids, SDS, and bacterial chromosomal DNA.

Prior to isolation of the plasmid DNA in a GenElute Miniprep Binding Column, the column was prepared for maximal binding of DNA to the membrane by the addition of 500 μ l Column Preparation Solution, followed by centrifugation at 14 000 rpm for 1 minute. Once the flow-through liquid from the column had been discarded from the collection tube, the cleared lysate produced following neutralisation was transferred to the miniprep column and centrifuged at 14 000 rpm for 1 minute to bind the plasmid DNA to the membrane. The flow-through liquid was discarded and 500 μ l Optional Wash Solution was added to the column, which was then centrifuged at 14 000 rpm for 1 minute. The flow-through liquid was discarded and, in order to remove residual salt and contaminants introduced during the column load, the column was washed by the addition of 750 μ l diluted Wash Solution and centrifugation at 14 000 rpm for 1 minute. The flow-through was discarded and the column was centrifuged again at 14 000 rpm for 2 minutes to remove excess ethanol left by the Wash Solution. The column was transferred to a sterile 1.5 ml microcentrifuge tube and 100 μ l best quality water was added to the column membrane to absorb for 5 minutes. Following centrifugation at 14 000 rpm for 1 minute, the purified plasmid DNA was eluted into the tube for storage at -20°C.

The negative control of LB and kanamycin used during culturing of the recombinant *E. coli* cells was subsequently used as a miniprep negative control to validate the results.

The purified DNA plasmids were analysed for the presence of the correct PCR product inserts by restriction enzyme analysis. Since the TOPO[®] Cloning site into which the PCR product ligates is situated immediately between two *EcoRI* cleavage sites within each plasmid vector, *EcoRI* enzymatic restriction of the plasmid results in the generation of two DNA fragments – a 3.5 kb fragment and a fragment with a size equivalent to that of the PCR product insert. As such, restriction analysis reveals the success of cloning by determining the presence and size of PCR product inserts.

Each restriction reaction mix consisted of 1 μ l Buffer H, 1 μ l *EcoRI* restriction enzyme (Promega, Madison, WI) (5' AGG[▼]CCT 3' restriction site), 3 μ l best quality water, and 5 μ l plasmid DNA. The reagents were mixed by pipetting in a 0.5 ml microcentrifuge tube and were incubated at 37°C for 2 hours to ensure optimal enzymatic activity. The entire 10 μ l volume of the restriction reaction mix was then electrophoresed with 7.5 μ l bromophenol blue loading dye adjacent to both a 1 kb and 100 bp DNA ladder (Promega, Madison, WI) on an ethidium bromide-stained 1% agarose gel at ~100 V for 2 hours. Bands of the appropriate sizes (3.5 kb and 3.2 kb, or 3.5 kb and 541 bp) were visualised on the gel under ultraviolet light and corresponded to positive clones.

2.6 GENOTYPING OF HBV DNA

Using only the S gene of HBV, the virus may be accurately classified into one of the eight genotypes, A to H, by analysis of restriction fragment length polymorphisms (RFLPs). Following restriction of the S gene DNA sample with particular restriction endonucleases, the DNA is cut into a precise mixture of fragments that form a reproducible pattern of bands when subjected to agarose gel electrophoresis. By comparing the pattern produced after restriction of the experimental DNA sample with those formed by the S genes of the eight genotypes, the genotype of the sample can be deduced. Such differences in pattern, or RFLPs, result from differences in the positions of restriction sites, and thus from differences in the sequences, between genotypes.

The genotype of each of the full-length genome clones and S gene clones from both the serum and liver samples was determined by RFLP analysis [Lindh *et al.*, 1997]. In order to isolate the HBV DNA from the purified recombinant plasmid DNA prior to genotyping, the segment of the HBV S gene between nucleotides 256 and 796 was amplified using the second round PCR protocol described in Section 2.3.2. For the first genotyping reaction, 10 μ l of the PCR product was mixed with 2 μ l 10X Buffer B, 0.2 μ l bovine serum albumin (BSA), 7.3 μ l best quality water, and 0.5 μ l *Hinf*I restriction enzyme (Promega, Madison, WI) (5' G[▼]ANTC 3' restriction site) in a 0.5 ml microcentrifuge tube. A drop of sterile mineral oil was added to the surface of the mixture to prevent evaporation, which was then incubated at 37°C for 3 hours. In a separate reaction, 10 μ l of the PCR product was mixed with 2 μ l 10X NEBuffer 1, 7.5 μ l best quality water, and 0.5 μ l *Tsp*509I restriction enzyme (New England Biolabs, Beverly, MA) (5' [▼]AATT 3' restriction site) in a 0.5 ml microcentrifuge tube. A drop of sterile mineral oil was added to the surface of the

mixture, which was subsequently incubated at 65°C for 3 hours. Following incubation, each 20 μ l sample was electrophoresed with 5 μ l bromophenol blue loading dye adjacent to a 1 kb and 100 bp DNA ladder (Promega, Madison, WI) on an ethidium bromide-stained 3% composite agarose gel at 40 V overnight. The DNA restriction patterns were visualised under ultraviolet light and compared with those published [Lindh *et al.*, 1997].

An additional genotyping reaction was performed on the full-length genome clones to distinguish subgenotype A1 from A2. A single round PCR using primers specific to parts of the S and P genes of HBV was performed in order to amplify this region of the genome from the recombinant plasmids (*Table 2.3*).

Table 2.3 Oligonucleotide primers used for the PCR amplification of parts of the S and P genes of HBV for A1 versus A2 genotyping

Primer	Sequence	Position* (nt)	Size† (bp)
521 (+)	5' CCTGCACGACTCCTGCTCAA 3'	522-541	671
1192 (-)	5' CGTCAGCAAACACTTGGCAC 3'	1192-1173	

Abbreviations: (+), sense; (-), antisense

* Nucleotide position of HBV *adw* genome (GenBank accession number AY233276), where position 1 is the *Eco*RI cleavage site.

† Size of the PCR amplicon in base pairs.

The final 50 μ l reaction mix for the PCR consisted of 0.2 μ l, 0.014 U/ μ l Expand High Fidelity Enzyme (Roche Applied Science, Germany); 5 μ l 1X Expand High Fidelity buffer (including 1.5 mM MgCl₂); 1 μ l, 200 μ M of each of the four dNTPs; 2.5 μ l, 1 μ M of each of the primers; 5 μ l (10% vol/vol) DNA; and 30.8 μ l best quality water. The

concentrations are those of the reagents in the final volume. PCR was performed in an Eppendorf Mastercycler[®] Gradient programmable thermal cycler (Eppendorf, Hamburg, Germany) using a cycle profile of 94°C for 3 minutes as an initial activation step, followed by 40 cycles of 94°C for 45 seconds (denaturation), 60°C for 1 minute (annealing), and 72°C for 90 seconds (extension), followed by a final extension step at 72°C for 7 minutes. PCR products were kept at 4°C until needed.

The A1 versus A2 genotyping reaction involved the addition of 10 μ l of the PCR product to 7.5 μ l best quality water, 2 μ l 10X Buffer B, and 0.5 μ l *Stu*I restriction enzyme (Promega, Madison, WI) (5' AGG[▼]CCT 3' restriction site) in a 0.5 ml microcentrifuge tube. After a drop of sterile mineral oil had been added to the surface of the mixture, it was incubated at 37°C for 1 hour to promote optimal enzymatic activity. Following incubation, each 20 μ l sample was electrophoresed and analysed in the manner described for the previous genotyping method.

2.7 NUCLEOTIDE SEQUENCING OF HBV DNA

The positive clones containing the full-length HBV genome (3.2 kb) and those containing the HBV S gene (541 bp) were sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa, for automated sequencing of the inserts. Such direct sequencing is based on the Sanger dideoxynucleotide chain termination DNA sequencing method, which uses PCR to incorporate fluorescent dye-labelled dideoxynucleotides (ddNTPs) into a

population of oligonucleotides synthesised from a single strand of the target DNA. Since the final PCR reaction mix contains oligonucleotides of different lengths, each terminating in one of the four uniquely labelled ddNTPs and designating each nucleotide in the target DNA sequence, high-resolution electrophoretic separation of the oligonucleotides on a polyacrylamide gel, followed by laser detection of the characteristic wavelength of fluorescence emitted by each ddNTP, allows for computer analysis of the DNA sequence.

The BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) was used by Inqaba Biotec to prepare the clones for automated sequencing on a Spectrumedix SCE2410 genetic analysis system with 24 capillaries (SpectruMedix LLC, State College, PA), using HBV-specific primers (*Table 2.4*). All sequences were analysed in both the forward and reverse directions.

Table 2.4 Oligonucleotide primers used for sequencing the full-length genome and S gene of HBV

Region [*]	Primer	Sequence	Position [†] (nt)	Annealing Temp. (°C)
<i>Full-Length</i>				
S	455 (+)	5' CAAGGTATGTTGCCCCGTTTG 3'	455-474	55
X	1418 (+)	5' CCTTTGTTTACGTCCCCGTCGG 3'	1418-1438	59
PreC/C	1822 (+)	5' TTTTTCACCTCTGCCTAATCA 3'	1821-1841	58
P & C	2409 (+)	5' TCTCAATCGCCGCGTCGCAGAAGATCTCAA 3'	2408-2437	66
P & PreS1	2800 (+)	5' CAGGTAGCGCCTCATTTTGTGGGTCACCATATTCT 3'	2800-2834	66
PreS1, 2 & S	270 (-)	5' AGAGAAGTCCACCACGAGTCTAGA 3'	270-247	65
P & S	1011 (-)	5' CAAAAGACCACAATTCTTTGACATACTTTCCAAT 3'	1011-977	66
P	1327 (-)	5' CGATAAGTTTGTCTCCAGACCGGCTGC 3'	1327-1301	66
X & PreC	1822 (-)	5' AAAAAGTTGCATGGTGCTGG 3'	1825-1806	58
X & PreC/C	1968 (-)	5' GTCAGAAGGCAAAAACGAGAG 3'	1966-1946	58
P & PreS1	2398 (-)	5' GCGAGGCGAGGGAGTTCTTCTTC 3'	2398-2376	56
<i>S Gene</i>				
S	P7 (+)	5' GTGGTGGACTTCTCTCAATTTTC 3'	256-278	53
S	P8 (-)	5' CGGTATAAAGGGACTCACGAT 3'	796-776	53

Abbreviations: (+), sense; (-), antisense.

^{*} Region of HBV genome amplified for sequencing.

[†] Nucleotide position of HBV *adw* genome (GenBank accession number AY233276), where position 1 is the *EcoRI* cleavage site.

2.8 SEQUENCING DATA AND PHYLOGENETIC ANALYSIS

To identify any mutations, the genomic or S gene sequence obtained from each of the serum and liver sample clones was compared with the corresponding sequence of numerous HBV isolates within each of the eight genotypes obtained from GenBank. Only mutations that occurred in both the forward and reverse sequences were recorded.

The multiple sequences were aligned and edited manually in GeneDoc [Nicholas *et al.*, 1997] and then fed into PHYLIP version 3.5c, a phylogeny inference package used for phylogenetic analysis [Felsenstein, 2004]. This latter type of analysis makes use of variations between the genes of HBV isolates to illustrate their evolutionary relationships. DNADIST and NEIGHBOUR (neighbour-joining), a phylogenetic analysis algorithm that creates a distance (dissimilarity) matrix by step-wise clustering, were used consecutively to generate a dendrogram or phylogenetic tree. To test the statistical reliability of the tree by correcting for sampling errors, bootstrap analysis of 1 000 replicate datasets was performed using DNADIST and NEIGHBOUR. Finally, the tree was visualised using the TreeView Win 32 software program [Page, 1996].

Chapter 3: RESULTS

3.1 DETECTION OF AMPLIFIED DNA PRODUCTS

3.1.1 Full-Length Genome Amplicons

The complete, or full-length, genome of the HBV DNA extracted from the patient's serum was successfully amplified using single round PCR, and was indicated by the presence of 3.2 kb DNA bands on the agarose gel (*Figure 3.1*).

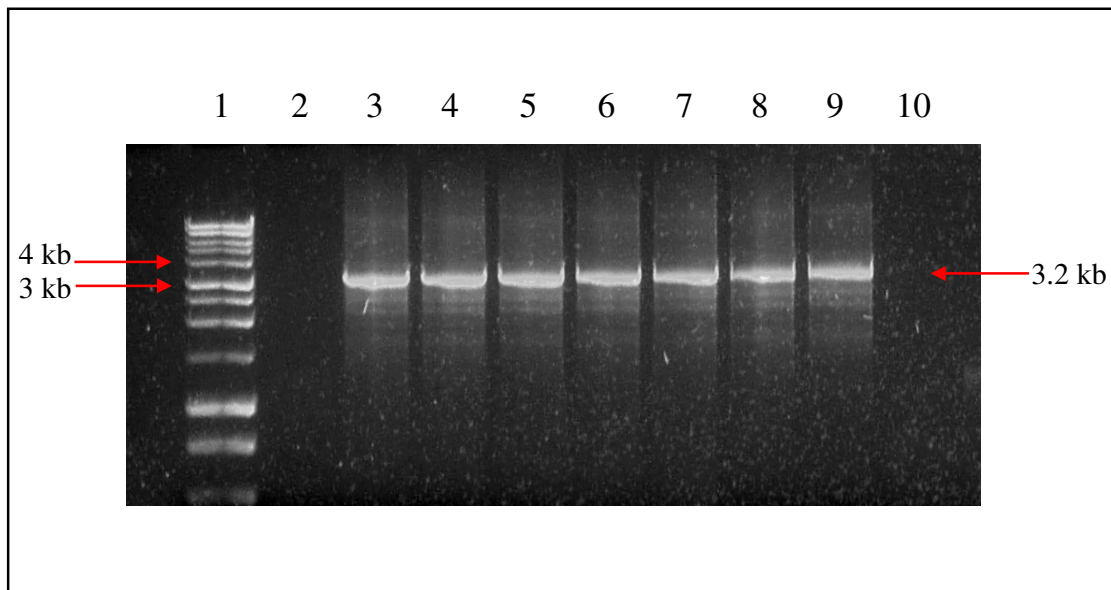


Figure 3.1 Detection of the amplified full-length HBV genome as a 3.2 kb PCR product on an ethidium bromide-stained 1% agarose gel.

Lane 1 = 1 kb molecular weight marker

Lanes 2 & 10 = water controls

Lanes 3-9 = HBV DNA extracted from patient's serum

3.1.2 Surface Gene Amplicons

Using nested PCR, the surface gene of the HBV genome was successfully amplified from both the serum- and liver tissue-extracted HBV DNA of the patient, as shown by the bands of 541 bp on the agarose gel (*Figure 3.2*).

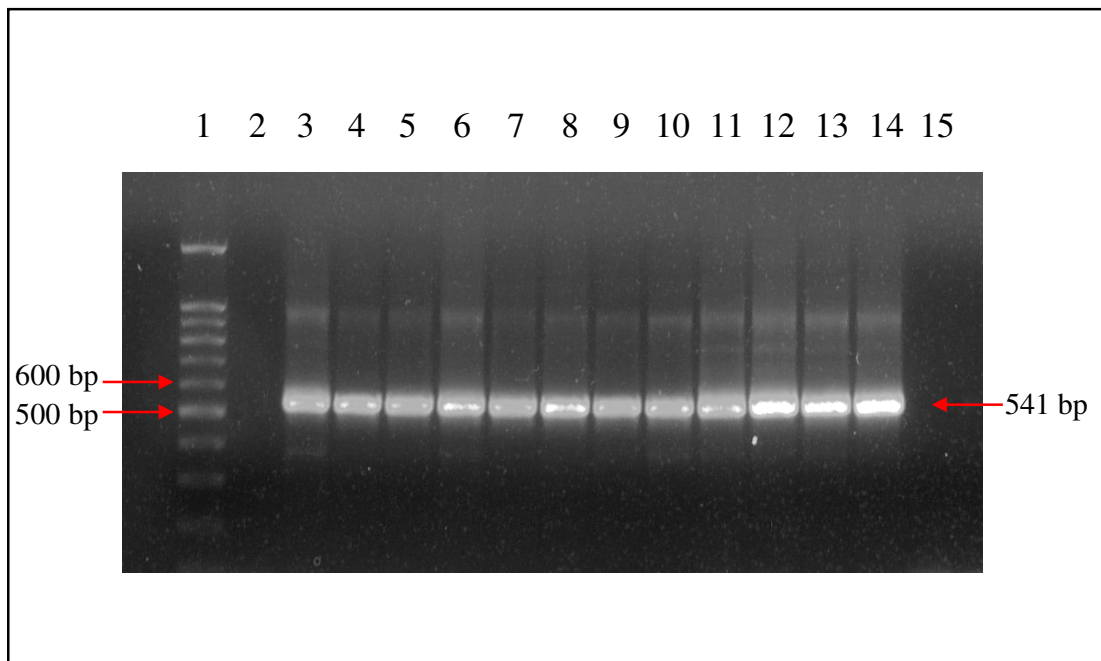


Figure 3.2 Detection of the amplified HBV surface gene as a 541 bp PCR product on an ethidium bromide-stained 1% agarose gel.

Lane 1 = 100 bp molecular weight marker

Lanes 2 & 15 = water controls

Lanes 3-14 = HBV DNA extracted from patient's serum or liver

3.2 IDENTIFICATION OF HBV POSITIVE CLONES

The full-length PCR products from the serum, and the surface gene products from the serum and the liver tissue, were all successfully cloned into the 3.5 kb pCR[®]-XL-TOPO[®] plasmid vector using TOP10 *E. coli* cells. Following isolation of the recombinant plasmids, and using *Eco*RI restrictions and RFLP analysis, 48 full-length HBV positive clones were identified by their restriction pattern of 3.5 kb, 1.8 kb, and 1.4 kb bands on the agarose gel (*Figure 3.3*). Similarly, surface gene positive clones displayed a restriction pattern of 3.5 kb and 541 bp bands on the agarose gel, thereby enabling the identification of 130 serum clones and 57 liver tissue clones (*Figure 3.4*).

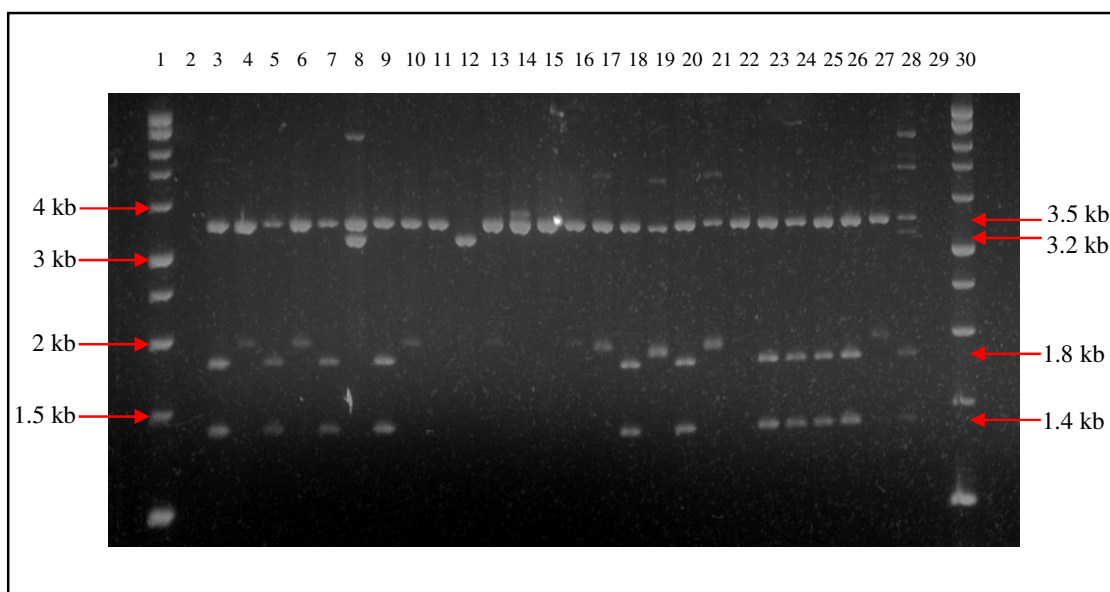


Figure 3.3 Identification of full-length HBV positive clones as 3.5 kb, 1.8 kb, and 1.4 kb DNA bands on an ethidium bromide-stained 1% agarose gel.

- | | |
|--------------------------------------|---|
| Lanes 1 & 30 | = 1 kb molecular weight markers |
| Lanes 2 & 29 | = water controls |
| Lanes 3, 5, 7-9, 18, 20, 23-26, & 28 | = positive clones of full-length HBV from patient's serum |
| Lanes 4, 6, 10-17, 19, 21, 22, 27 | = unsuccessful clones containing no HBV DNA inserts |

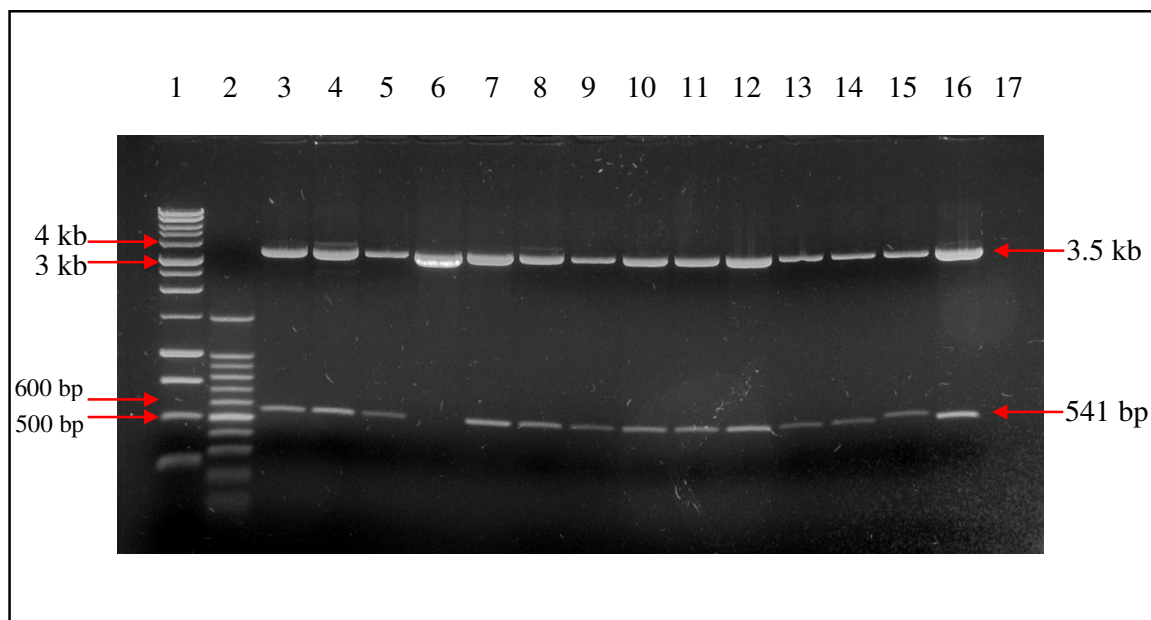


Figure 3.4 Identification of HBV surface gene positive clones as 3.5 kb and 541 bp DNA bands on an ethidium bromide-stained 1% agarose gel.

Lane 1 = 1 kb molecular weight marker

Lane 2 = 100 bp molecular weight marker

Lanes 3-16 = positive clones of HBV surface gene from patient's serum or liver tissue

Lane 17 = water control

3.3 HBV GENOTYPING BY RFLP ANALYSIS

3.3.1 Genotyping A to H

The region of the S gene between nucleotides 256 and 796 was successfully amplified from each of the full-length genome clones and the S gene clones, as shown by the presence of 541 bp PCR products on the agarose gel (*Figure 3.2*). Following enzymatic restriction of the PCR products by *HinfI* and *Tsp509I* in separate reactions, electrophoresis

of each PCR product resulted in fragments of 274 bp, 252 bp, and 15 bp in size, and 207 bp, 126 bp, 109 bp, 47 bp, 36 bp, and 16 bp in size, respectively. However, only fragments larger than 50 bp were visualised on the agarose gel (*Figure 3.5*). According to Lindh *et al.* (1997), such an RFLP pattern is characteristic of HBV genotype A.

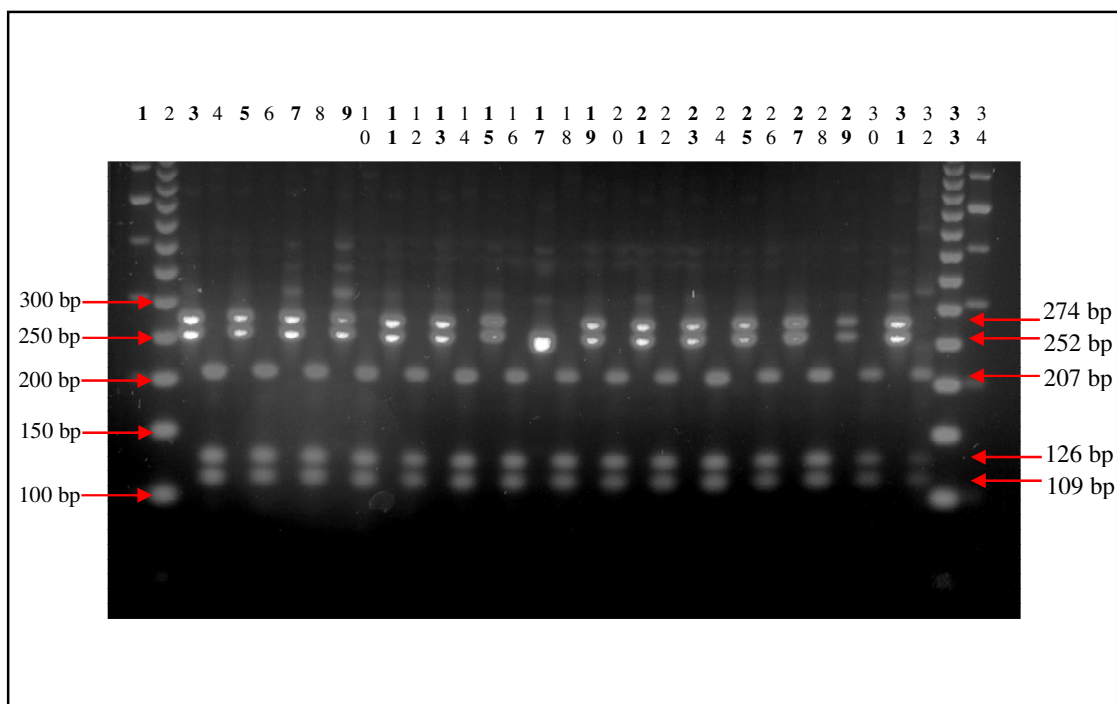


Figure 3.5 Restriction fragment length polymorphism (RFLP) analysis of the HBV surface gene (nt 256-796) amplified from all of the serum and liver tissue clones, showing the characteristic pattern of genotype A on an ethidium bromide-stained 3% composite gel.

Lanes 1 & 34	= 100 bp molecular weight markers
Lanes 2 & 33	= 50 bp molecular weight markers
Lanes 3-31 (odd numbers)	= PCR products restricted with <i>HinfI</i>
Lanes 4-32 (even numbers)	= respective PCR products restricted with <i>Tsp509I</i>

3.3.2 Subgenotyping A1 versus A2

The single round S and P region PCR performed on the full-length genome clones resulted in the production of 671 bp DNA products, as shown following agarose gel electrophoresis. Enzymatic restriction of each PCR product by *StuI* resulted in the production of two DNA fragments, sized 593 bp and 78 bp, corresponding to the RFLP pattern representative of subgenotype A1 (Figure 3.6).

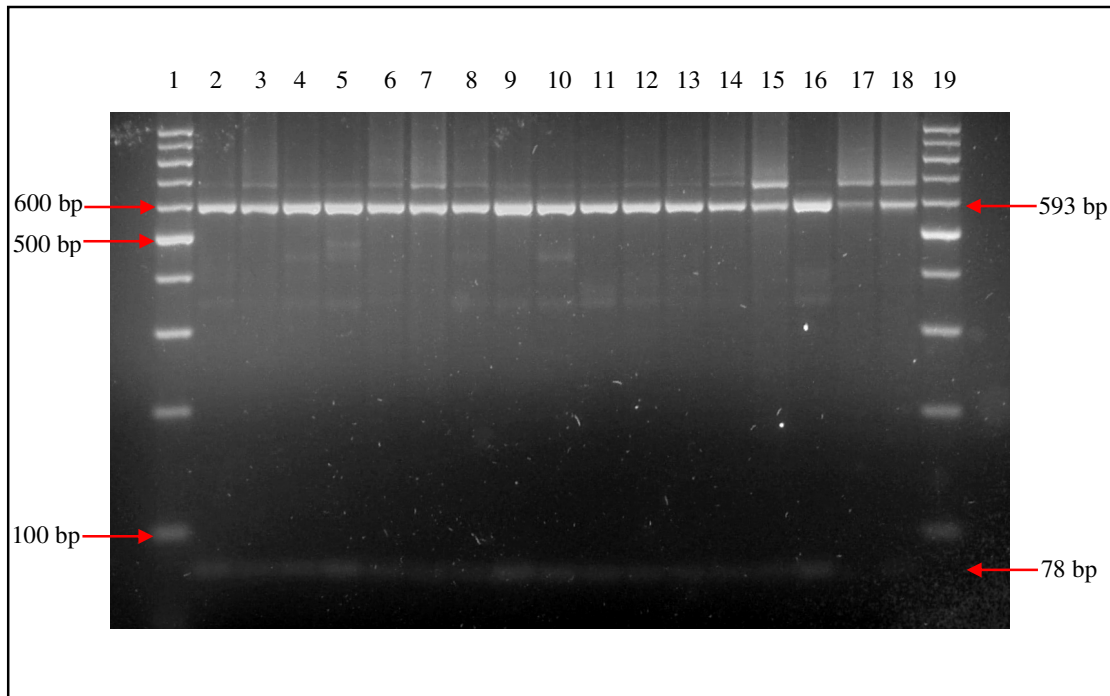


Figure 3.6 Restriction fragment length polymorphism (RFLP) analysis of the HBV surface and polymerase gene (nt 522-1192) amplified from the serum full-length genome clones, showing the characteristic pattern of subgenotype A1 on an ethidium bromide-stained 3% composite gel.

Lanes 1 & 19 = 100 bp molecular weight markers

Lanes 2-18 = full-length HBV genome clones from patient's serum

3.4 DNA SEQUENCING ANALYSIS

Automated direct sequencing of the complete genome amplified from the patient's serum-extracted HBV isolates revealed a wild-type S gene within a 3 215 bp genome (GenBank accession number AY903452, awaiting release). In contrast to all other genotype A isolates, which possess a six nucleotide insert between nucleotides 2 353 and 2 360 in the core region, and hence have genome lengths of 3 221 bp, the patient's HBV isolates lacked the short insert, as do the seven other genotypes (*Figure 3.7*). However, alignment of the complete genome sequence with multiple corresponding sequences within each of the eight genotypes confirmed the isolate to be that of genotype A.

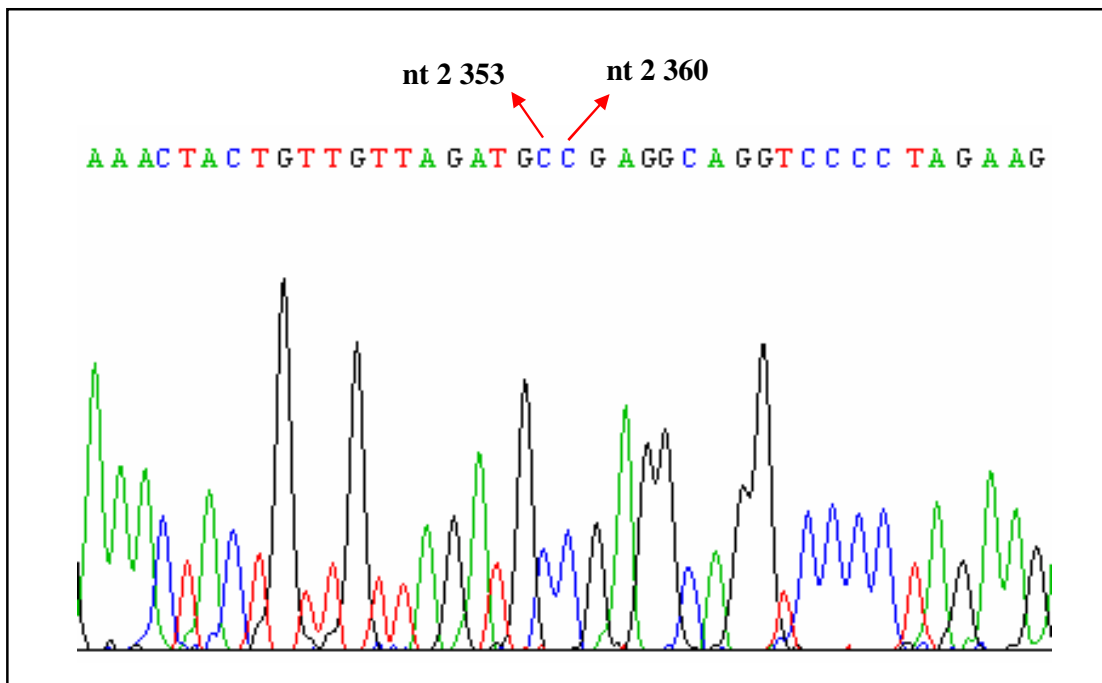


Figure 3.7 Chromatogram produced following automated sequencing of the core gene of the patient's HBV isolates, visualised using Chromas Version 1.45 [McCarthy, 1998], showing the lack of the six nucleotide insert – 5' CGAGAT 3' – between nt 2 353 and 2 360 according to GenBank accession number AY233276.

Sequence analysis of each of the open reading frames identified amino acids that are unique to subgenotype A1 [Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Kimbi *et al.*, 2004]. Within the preS1 region overlapping the spacer of the polymerase, the subgenotype A1-specific residues were Gln⁵⁴, Val⁷⁴, Ala⁸⁶, and Val⁹¹; in the preS2 region, residues included Leu³², Val³⁵, Ser⁴⁷, and Pro⁵⁴; differentiating amino acids in the polymerase region were His⁸⁷, Asn¹²⁰, Ser¹²¹, His¹⁸², Phe²²⁰, Thr²³⁶, Ser²⁵¹, Gly²⁶⁸, Ala²⁷¹, Ser²⁷³, Ser³⁰⁸, Phe³⁰⁹, Arg³¹⁵, Gln³³⁴, Lys³³⁸, and Leu⁴⁷⁷; and within the X region, residues Ser¹¹, Ala³¹, and Ser⁴⁷, were characteristic of subgenotype A1. Sequencing analysis additionally revealed the silent G1888A nucleotide mutation within the precore/core open reading frame, which stabilises the encapsidation signal and is unique to subgenotype A1.

The serological subtype of the isolate was deduced from the genome sequence to be *adw2*, by the presence of Lys¹²², Pro¹²⁷, Phe¹³⁴, Thr¹⁴³, Ala¹⁵⁹, and Lys¹⁶⁰ within the S region.

3.4.1 Full-Length Genome Clones from Serum

Sequencing the S gene (nt 256-796) of the 48 full-length genome clones revealed 42 to be wild-type isolates, while six (13%) displayed unique or uncommon non-synonymous (missense) mutations within the region of the gene encoding the major hydrophilic region (MHR) of the HBsAg (aa 100-160), when compared to the corresponding sequences of various HBV isolates obtained from GenBank (*Figure 3.8*). Of these mutant isolates, three displayed mutations within the region of the S gene encoding the second loop of the *a* antigenic determinant (aa 139-147), the major neutralising epitope of the HBsAg protein.

One of the clones, designated SerClone 16, had an A-to-G point mutation at nt 581, which resulted in a Thr to Ala substitution at residue 143. SerClone 17 and SerClone 18, the remaining mutant isolates, both displayed A-to-G transitions at nt 590 that caused the mutation of Asn¹⁴⁶ into Asp¹⁴⁶ (*Table 3.1*).

In addition to variation within the major epitopes of the *a* determinant (aa 124-147), amino acid substitutions in the adjacent antigenic regions, although relatively uncommon, may also have a considerable effect on its antigenicity. SerClone 17 contained a second mutation that led to variation within these outer regions of the *a* determinant: an A-to-G transition at nt 503 converted Ser¹¹⁷ into Gly¹¹⁷. Sequencing additionally showed the remaining three mutant clones, SerClone 4, 5, and 6, as having G-to-A point mutations at nt 458, which resulted in Gly¹⁰² being substituted with Ser¹⁰² in the region of the MHR adjacent to the *a* determinant (*Table 3.1*).

3.4.2 Surface Gene Clones from Serum

Of the 130 HBV clones containing surface gene inserts (nt 256-796), sequencing revealed 119 isolates as being wild-type, while 11 (8.5%) displayed non-synonymous mutations within the region of the gene encoding the MHR of the HBsAg (*Figure 3.8*). Seven such isolates were each found to possess a mutation within the major epitope region of the *a* determinant between residues 124 and 147. SerClone 9 had a C-to-T point mutation at nt 534, which resulted in a Pro to Leu substitution at aa 127. SerClone 10 had an Ala to Thr substitution at aa 128 due to a G-to-A transition at nt 536. Sequencing revealed both

SerClone 11 and SerClone 12 as having a T-to-C mutation at nt 566 that caused the conversion of Cys¹³⁸ into Arg¹³⁸. Similarly, a T-to-C point mutation at nt 569 within SerClone 13 resulted in a Cys to Arg substitution at aa 139. Also effecting changes within the second loop of the α determinant, an A-to-G transition at nt 576 within SerClone 14 caused the substitution of Lys¹⁴¹ with Arg¹⁴¹, and at nt 579 within SerClone 15, a C-to-T mutation resulted in the conversion of Pro to Leu at residue 142 (*Table 3.1*).

Immediately adjacent to the second loop of the α determinant, and thus still important for its antigenicity, the mutation of Thr¹⁴⁸ to Ala¹⁴⁸ due to an A-to-G transition at nt 596 was found within SerClone 19. The remaining three mutant isolates contained mutations located outside the α determinant, but still within the MHR. SerClone 3 had a G-to-C transversion mutation at nt 459, causing a Gly¹⁰² to Ala¹⁰² substitution, whereas SerClone 7, in common with SerClone 4-6, displayed a G-to-A mutation at nt 458 that changed Gly¹⁰² to Ser¹⁰². A T-to-C point mutation at nt 471 within the sequence of SerClone 8, resulted in the substitution of Val with Ala at aa 106. Finally, in addition to the K141R amino acid mutation, SerClone 14 had Met¹⁰³ substituted by Ile¹⁰³ due to the point mutation of G-to-A at nt 463 (*Table 3.1*).

3.4.3 Surface Gene Clones from Liver Tissue

Sequencing analysis of the S gene inserts (nt 256-796) within the 57 clones from the liver tissue revealed 55 to be wild-type and two (3.5%) as displaying missense mutations within the region of the gene encoding the MHR of the HBsAg (*Figure 3.8*). As did SerClone 14,

LivClone 20 had a G-to-A mutation at nt 463 that resulted in the substitution of Met¹⁰³ by Ile¹⁰³. Additionally, this clone possessed a G-to-A point mutation at nt 570 that caused a Cys to Tyr change at aa 139 within the second loop of the *a* determinant. Also within this region, LivClone 21, displaying a G-to-A transition at nt 584, had a substitution of Asp¹⁴⁴ by Asn¹⁴⁴ (*Table 3.1*).

For means of clarity, the variations within the important epitopes of the *a* determinant region of the HBsAg (aa 124-147) of the serum- and liver-derived clones are shown schematically in *Figure 3.9*, which emphasises the fact that three occur in the first hypothesised loop of the *a* determinant (aa 124-138, a less conserved region), while seven are situated in the highly conserved second loop (aa 139-147). Additionally, a summary of the mutations occurring within the MHR is provided in *Table 3.1*.

As can be seen in *Figure 3.8*, natural variation of certain amino acids was common to all isolates and, as shown by comparisons with the corresponding amino acids in the sequences obtained from GenBank (light blue shading), correlates with subtype- and genotype-defining mutations that have been selected over centuries of infection. The alignment clearly shows that all of the HBV clones display features of genotype A, both at the genotype and serological subtype levels.

	45	60	75	90	105	120
SerClone1	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone2	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	VFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone3	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	AMLPVCPLIPG	STTTSTGP
SerClone4	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	SMLPVCPLIPG	STTTSTGP
SerClone5	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	SMLPVCPLIPG	STTTSTGP
SerClone6	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	SMLPVCPLIPG	STTTSTGP
SerClone7	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	SMLPVCPLIPG	STTTSTGP
SerClone8	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPACPLIPG	STTTSTGP
SerClone9	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone10	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone11	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone12	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone13	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone14	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GILPVCPLIPG	STTTSTGP
SerClone15	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone16	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone17	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTCTGP
SerClone18	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
SerClone19	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
LivClone20	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GILPVCPLIPG	STTTSTGP
LivClone21	: PPVCLGQNSQS	PTSNHSPTS	CPPNCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
GenotypeA	: SPVCLGQNSQS	PTSNHSPTS	CPPICPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
GenotypeA1	: SPVCLGQNSQS	PTSNHSPTS	CPPICPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
GenotypeB	: TPVCLGQNSQS	QISHSPT	CCPPICPGYRWMCLRRFI	IFLCIILLCLIFLLVLLDYQ	GMLPVCPLIPG	SSTTSTGP
GenotypeC	: TPKCPGQNSQS	PTSNHSPTS	CPPTCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
GenotypeD	: TTVCLGQNSQS	PTSNHSPTS	CPPTCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	SSTTSTGP
GenotypeE	: APVCLGQNSQS	PTSNHSPTS	CPPICPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	SSTTSTGP
GenotypeF	: LPGCPGQNSQS	PTSNHLPTS	CPPTCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP
GenotypeG	: VPVCPGLNSQS	PTSNHSPTS	CPPTCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	SSTTSTGP
GenotypeH	: PPGCPGQNSQS	PISNHLPTS	CPPTCPGYRWMCLRRFI	IFLEIILLCLIFLLVLLDYQ	GMLPVCPLIPG	STTTSTGP

Continued over page...

	121	135	150	165	180	195								
SerClone1	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone2	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone3	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone4	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone5	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone6	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone7	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone8	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone9	: CKTCTT	LAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone10	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone11	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone12	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone13	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone14	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone15	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone16	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone17	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone18	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
SerClone19	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
LivClone20	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
LivClone21	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeA	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeA1	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeB	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeC	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeD	: CRTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeE	: CRTCTT	LAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeF	: CKTCTT	LAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeG	: CKTCTT	PAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY
GenotypeH	: CKTCTT	LAQGN	SMFPSCCCTKPTDGNCT	CIPI	SSWAF	AKYLW	EWASV	RFS	SWLS	LLV	PFVQW	FVGLS	PTVWLS	SAIWMMWY

Figure 3.8 Amino acid sequence of part of the HBsAg (aa 45-200), deduced from the nucleotide sequence of HBV S gene DNA (nt 287-754) from the patient's serum- and liver-derived HBV clones, 1-21. The corresponding sequences of the eight HBV genotypes, A to H, including subgenotype A1, are aligned for comparative purposes. Dark blue shading indicates a unique or uncommon amino acid variation, light blue shading indicates a subtype- or genotype-defining variation, pink shading represents the consensus sequence, and yellow shading highlights the region of the *a* determinant between residues 124 and 147 containing the major antigenic epitopes. Twelve clones displayed mutations within this important latter region.

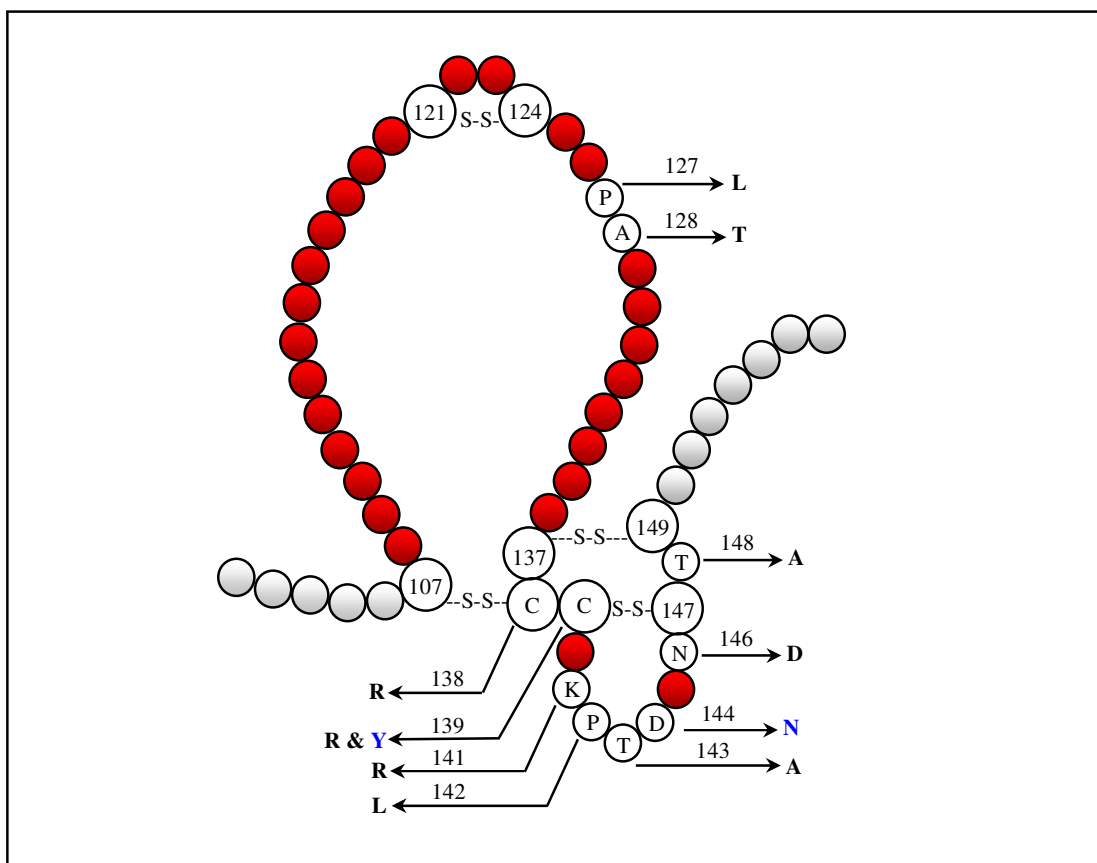


Figure 3.9 Schematic diagram of the HBsAg α determinant (red circles; aa 107-147) showing single amino acid mutations (arrows) found between aa 124 and 148 in several clones of HBV obtained from the serum (black) and liver tissue (blue) of the vaccinated South African patient. Large circles represent cysteine residues connected by disulphide (S-S) bonds, according to the cysteine web model of the HBsAg.

Table 3.1 Summary of mutations found within the region of the S gene encoding the MHR (aa 99-168) of the HBsAg from HBV clones

Source of HBV	HBV Clone	Nucleotide Mutation	Amino Acid Mutation
Serum	SerClone 3 (W.P35)	G 459 C	G 102 A
	SerClone 4 (W 2.21)	G 458 A	G 102 S
	SerClone 5 (W 2.26)	G 458 A	G 102 S
	SerClone 6 (W 2.27)	G 458 A	G 102 S
	SerClone 7 (W.P6)	G 458 A	G 102 S
	SerClone 8 (W.P32)	T 471 C	V 106 A
	SerClone 9 (W.P50)	C 534 T	P 127 L
	SerClone 10 (W.P1)	G 536 A	A 128 T
	SerClone 11 (W.P2)	T 566 C	C 138 R
	SerClone 12 (W.P20)	T 566 C	C 138 R
	SerClone 13 (W.P160)	T 569 C	C 139 R
	SerClone 14 (W.P135)	G 463 A A 576 G	M 103 I K 141 R
	SerClone 15 (W.P147)	C 579 T	P 142 L
	SerClone 16 (W 2.18)	A 581 G	T 143 A
	SerClone 17 (W 2.5)	A 503 G A 590 G	S 117 G N 146 D
	SerClone 18 (W 9)	A 590 G	N 146 D
	SerClone 19 (W.P61)	A 596 G	T 148 A
Liver	LivClone 20 (W.L34)	G 463 A G 570 A	M 103 I C 139 Y
	LivClone 21 (W.L3)	G 584 A	D 144 N

3.5 PHYLOGENETIC ANALYSIS

The dendrogram obtained by neighbour-joining phylogenetic analysis of the complete HBV nucleotide sequence amplified from the serum, and the corresponding sequences of 36 additional isolates obtained from GenBank, is shown in *Figure 3.10*. Based on analysis of the full-length sequence, and of those of the four individual open reading frames (data not shown), the patient's HBV isolates clustered with genotype A1, the predominant genotype amongst South African HBV isolates. It should be emphasised that bootstrap statistical analysis performed using 1 000 data sets supported the nodes of the dendrogram very well, as shown by the high percentage of bootstrap trees supporting those nodes. Since the clustering of the patient's HBV isolate into subgenotype A1 was well supported, conclusions could be made with relative confidence in its regard.

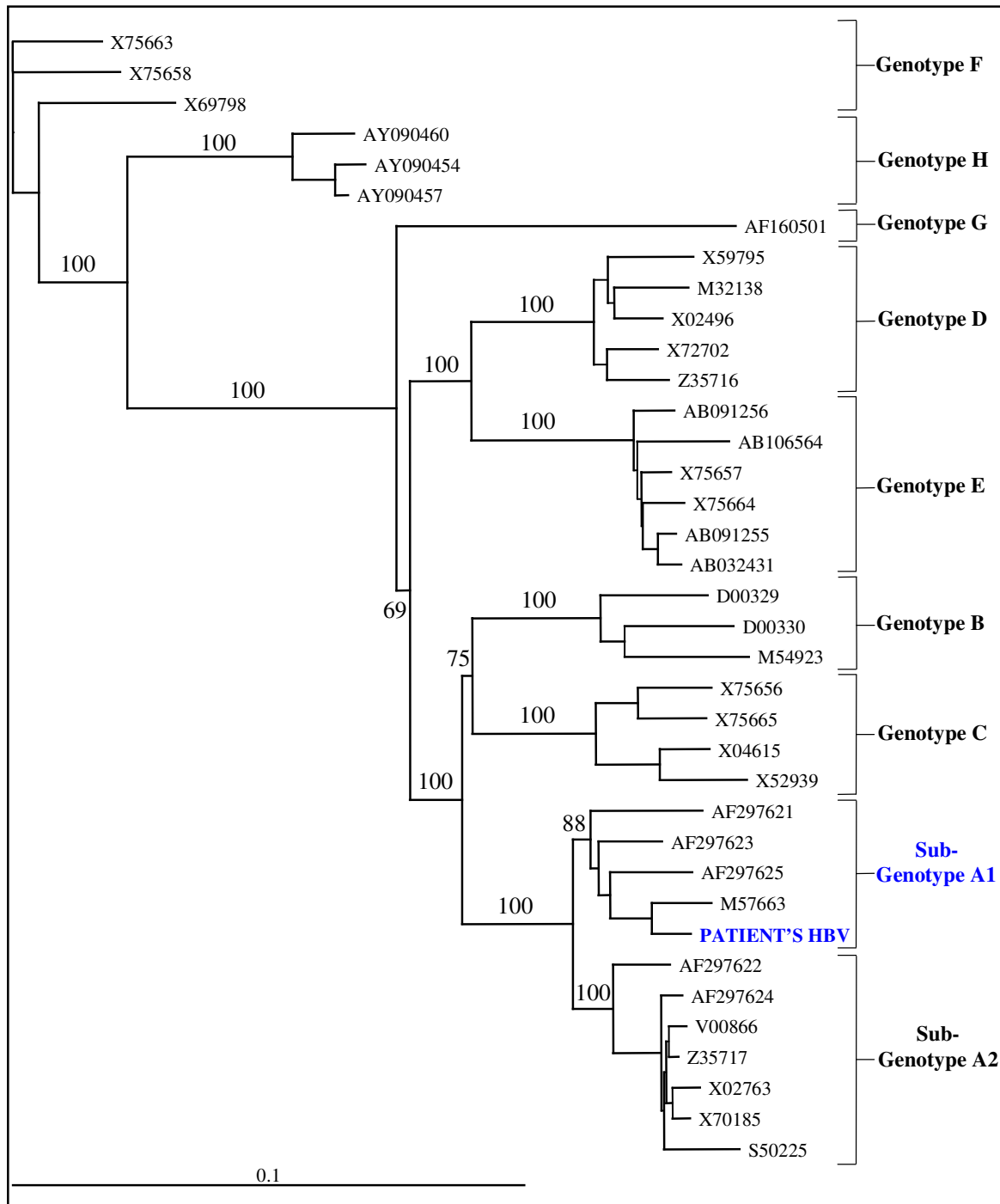


Figure 3.10 Dendrogram obtained by neighbour-joining phylogenetic analysis of HBV complete genome sequences rooted on genotype F, showing the relationship of the HBV isolate obtained from the vaccinated patient's serum (blue) to the eight HBV genotypes (sequences designated by GenBank accession numbers), where A1 and A2 are subgenotypes of genotype A. Inter-isolate distances were calculated by DNADIST using the Kimura two-parameter model with a transition to transversion ratio of 2 and a randomised input order of sequences. The horizontal line represents a 10% distance. Bootstrap statistical analysis was performed using 1 000 data sets and the numbers on the nodes indicate the percentage of occurrences.

Chapter 4: DISCUSSION

The 3.2 kb DNA genome of the hepatitis B virus is highly compact, yet, because of the overlapping nature of its four open reading frames, it is capable of encoding seven major proteins – polymerase, HBcAg, HBeAg, HBxAg, preS1 surface protein, preS2 surface protein, and HBsAg. Such a highly developed evolution has almost certainly been facilitated by the inclusion of an RNA replicative intermediate with an error-prone reverse transcription step in the viral life cycle [Brown *et al.*, 1992]. For this very reason, instead of the virus being relatively intolerant to change – as might be expected owing to the characteristics of its genome – HBV displays a wide variability and is able to adapt to changing immunological pressures, while still maintaining the integrity of its reading frames.

Of all the variation known to occur in the HBV genome, mutations within the surface, or S, gene are the most clinically relevant. These mutations may affect the immunogenicity of the HBsAg, particularly if the antigenic *a* determinant, against which the major neutralising antibodies are raised, is altered. Such mutants arise naturally following errors in replication and can subsequently become the dominant viral species in a vaccinated host as a result of the selective pressure of vaccine-induced antibodies. As such, these mutants are capable of evading the host immune response and causing infections in both unvaccinated and vaccinated individuals. It is important that the prevalence, variety, and antigenic significance of these S gene mutations occurring in HBV in endemic areas be recorded, as this may affect policy decisions relating to vaccine and diagnostic assay design.

In this study, the S gene of HBV from the first reported South African case of breakthrough HBV infection was analysed in order to identify mutations within the HBsAg that may have permitted viral escape from neutralisation by vaccine-induced anti-HBs. Such information should provide a comparison of South African HBV vaccine-escape mutants with those found in different geographical regions of the world.

HBV DNA was extracted from both the serum and liver tissue of the HBV-infected, vaccinated patient, which allowed for a comparison of viral strains present in the hepatocytes with those secreted into the blood stream. The protocols used for the extraction of HBV DNA from the serum and liver tissue samples were highly efficient, as high concentrations of viral DNA were obtained from both samples and repetition of the extraction procedures was unnecessary. However, the method used for preservation of the liver tissue in paraffin-wax caused the fragmentation of the 3.2 kb HBV DNA into ~650 bp segments, which was not preferential for downstream applications such as complete genome amplification.

Amplification of the complete HBV genome using the single-round PCR method described by Gunther *et al.* (1995) was convenient and efficient. The main concern regarding the use of PCR prior to genetic mutational analysis is the incorporation of errors into the DNA by *Taq* DNA polymerase during thermocycling and amplification. In this study, such a problem was minimised by the use of an Expand High Fidelity Enzyme mix, which combines the high 5'-3' polymerase activity of *Taq* DNA polymerase with the 3'-5' exonuclease proofreading activity of *Tgo* DNA polymerase to provide three times the fidelity and twice the yield of singular *Taq* polymerase. As such, the combination of

enzymes provides a low error rate of 8.5×10^{-6} nucleotide substitutions per site and therefore produces PCR amplicons displaying accurate sequences.

Following the use of various unsuccessful cloning techniques, including the PCR-ScriptTM Amp Cloning Kit (Stratagene, La Jolla, Ca), the pGEM[®]-T Easy Vector System Kit (Promega, Madison, WI), the QIAGEN[®] PCR Cloning Kit (QIAGEN GmbH, Hilden, Germany), and the Eukaryotic TA Cloning[®] Kit (Bidirectional) (Invitrogen, Carlsbad, CA), the full-length HBV genome and S gene amplicons were successfully cloned using the TOPO[®] XL PCR Cloning Kit. This latter kit is designed specifically for the cloning of long (3-10 kb) PCR products and was therefore ideal for use with the 3.2 kb full-length HBV genome amplicon. An additional advantage of the kit was the incorporation of an activated plasmid vector – one to which topoisomerase I is covalently bound. Such an enzyme binds to dsDNA at specific sites and cleaves the phosphodiester backbone after 5' CCCTT 3' in one strand. The energy from the broken phosphodiester backbone is conserved by the formation of a covalent bond between the 3' phosphate of the cleaved strand and Tyr²⁷⁴ of topoisomerase I. The bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, thereby reversing the reaction and releasing topoisomerase. To efficiently clone PCR products, this cloning kit exploits such a reaction by the inclusion of topoisomerase I cleavage sites within the cloning site of the vector.

Upon DNA sequencing, only one complete HBV genome sequence was obtained directly from the serum-extracted HBV DNA. Although 48 full-length genome clones were obtained, all were sequenced exclusively within the S gene, as full-length sequencing required the use of eleven oligonucleotide primers and was therefore expensive, time-consuming, and unnecessary considering that the S gene was the main region of interest.

Automated direct sequencing of the complete HBV genome amplified from the serum-extracted HBV isolates revealed a wild-type S gene sequence with no mutations within the *a* determinant-encoding region. Direct sequencing provides the sequence of the dominant strain within a mixed viral population. As such, these results showed that S gene mutant strains, if any, were in the minority. The dominant strain did, however, possess mutations in other regions of the genome. Upstream of the region in the S gene encoding the *a* determinant, two mutations were found to cause the amino acid mutation of S45P and I68N within the first hydrophilic loop of the surface protein (*Figure 3.8*). Forming a polar loop within the virion interior (*Figure 1.5*), this domain facilitates the attachment and envelopment of HBV nucleocapsids from the cytosol via a binding site between residues 35 and 46 [Ponsel and Bruss, 2003]. The mutation at amino acid 45 of a polar serine residue for a non-polar proline residue, which has a cyclic pyrrolidine side chain, would therefore be likely to affect the binding ability of the hydrophilic domain. In addition, the region between amino acids 40 and 49 contains a MHC class I-restricted T-cell epitope [Tai *et al.*, 1997], which could have led to the immune selection of S45P.

Within the open reading frames, sequence analysis revealed amino acid changes that were unique to subgenotype A1 [Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Kimbi *et al.*, 2004]. Additionally, the silent G1888A nucleotide mutation was found within the precore/core gene of the isolates and is characteristic of subgenotype A1. This mutation stabilises the viral encapsidation signal [Kramvis and Kew, 1998] and may affect core protein translation as a result of the introduction of an out-of-frame AUG start codon [Kimbi *et al.*, 2004].

Analysis of the restriction fragment length polymorphism patterns produced following restriction of the amplified S gene of each HBV clone, classified the isolates as genotype A (*Figure 3.5*). Similarly, subgenotyping RFLP analysis further classified the isolates as subgenotype A1 (*Figure 3.6*). Another accurate method of genotyping and determining evolutionary relationships, neighbour-joining phylogenetic analysis of the complete genome sequence of HBV isolated from the patient's serum clearly supported the clustering of the isolate with subgenotype A1 within genotype A (*Figure 3.10*). Moreover, the isolate displayed variations in the four open reading frames that are characteristic of subgenotype A1. It was unusual, however, in that it lacked the six nucleotide insert 5' CGAGAT 3' between nt 2 353 and 2 360 in the core region (*Figure 3.7*), which is common to all genotype A isolates but absent in the other seven genotypes. The absence of the insert gave rise to a complete genome length of 3 215 bp – the length characteristic of genotypes B, C, F, and H. Individual phylogenetic analysis of each of the isolate's open reading frames gave results that were consistent with that of the complete genome, proving that no recombination had occurred between the genes of the isolate and those of another genotype. The lack of a six nucleotide insert in the C gene of the isolate could therefore not be explained by a recombinational event. A subsequent search of the GenBank database revealed it to be a very rare isolate.

The serological subtype of the HBV could be deduced from the translated S gene sequencing data by the presence of subtype-specific amino acid variations. The patient's isolates possessed Lys¹²², Pro¹²⁷, Phe¹³⁴, Thr¹⁴³, Ala¹⁵⁹, and Lys¹⁶⁰ within the HBsAg (*Figure 3.8*) and were therefore subtype *adw2* – the common subtype amongst South African HBV genotype A isolates.

The amplified full-length genome of the patient's serum-extracted HBV was cloned and 48 clones were sequenced within the S gene (nt 256-796). Similarly, 130 clones of surface gene amplicons from the serum HBV were sequenced within the same region. Taken together, 17 clones displayed unique or uncommon non-synonymous (missense) mutations within the region of the gene encoding the major hydrophilic region of the HBsAg (aa 100-160), when compared to the corresponding sequences of various HBV isolates obtained from GenBank (*Figure 3.8*).

Of these mutant isolates, four displayed mutations within the region of the S gene encoding the less conserved first loop of the HBsAg *a* determinant (aa 107-138). One such clone had a P127L amino acid mutation – a subtype-defining variation specifying ω 4 that is commonly found in genotype D and E isolates and in all isolates of genotypes F and H. The substitution of Pro¹²⁷, which has a cyclic pyrrolidine side chain, by Leu¹²⁷, which has an aliphatic hydrocarbon side chain, causes the removal of a β -turn from the first loop of the *a* determinant [Norder *et al.*, 1992a] and therefore has a pronounced effect on the tertiary structure of the determinant. Because antigenicity is dependent on the conformational structure of this epitope, such variation could drastically affect the ability of antibodies to recognise and bind to the epitope for neutralisation. Antibodies have previously been shown to display a reduced reactivity against a peptide with a P127L amino acid mutation [Ijaz *et al.*, 2003], but since a linear peptide is not representative of the *a* determinant on HBsAg, it is unknown whether the variation could lead to vaccine escape of the virus. It may, however, have implications affecting serological assays.

Another of the mutant clones identified in this group had an A128T amino acid substitution, which appears to be a unique mutation, having not been reported in the

literature. The substitution of Ala¹²⁸, a residue with a non-polar, short aliphatic hydrocarbon side chain, by Thr¹²⁸, having an uncharged, polar, long hydroxlic side chain, would be likely to alter the tertiary structure of the protein at that point, potentially affecting the antigenicity of the *a* determinant.

Two clones displayed a C138R amino acid mutation within the first loop of the antigen. The replacement of Cys¹³⁸, which has a short, polar thiol (SH) side chain, with the long, positively charged, polar guanidino side chain of Arg¹³⁸ would result in a pronounced alteration of the tertiary structure of the *a* determinant, particularly because of the loss of the disulphide bond between Cys¹³⁸ and Cys¹⁰⁷ that helps to maintain the 'cysteine web' structure of the antigenic determinant. Disulphide bonds involving cysteine residues in the MHR of HBsAgs perform an essential role in the antigenic properties of the HBV envelope protein [Vyas *et al.*, 1972; Mangold and Streeck, 1993]. In addition, disulphide bonds are equally important in terms of establishing the appropriate tertiary structure necessary for surface proteins to form higher-order association complexes and for traversing the exocytic pathway [Huovila *et al.*, 1992; Antoni *et al.*, 1994]. Therefore, the mutation of these cysteine residues alters the structure of the protein as a result of incorrect protein folding, which may prevent its secretion from hepatocytes. Experiments have shown that Cys¹³⁸ is essential for both antigenicity and secretion of HBsAg [Mangold *et al.*, 1995], suggesting that C138R mutants may be able to evade vaccine-induced antibodies. Indeed, such a case of vaccine-escape caused by C138R HBV mutants has occurred in Germany [Sterneck *et al.*, 1998].

Of the 17 serum HBV clones identified displaying mutations within the MHR-encoding region, six had amino acid substitutions within the highly conserved second loop of the *a*

determinant (aa 139-147), which represents the major neutralising epitope of the HBsAg. Similar to the two clones containing C138R amino acid mutations, one of the six clones had a novel C139R substitution. Although Cys¹³⁹ is dispensable for HBsAg secretion, it is essential for antigenicity [Antoni *et al.*, 1994; Mangold *et al.*, 1995]. As such, the respective isolate would have been able to escape neutralisation by vaccine-induced antibodies.

Substitution experiments by Steward *et al.* (1993) confirmed the relevance of the antigenic second loop by demonstrating that residues 141-145 within the loop are essential for antibody binding. Therefore, in the clone containing the K141R amino acid variation, the substitution of the positively charged, polar butylammonium side chain of Lys¹⁴¹ for the positively charged, polar guanidino side chain of Arg¹⁴¹ would be expected to abolish the reactivity of the antigen to anti-HBs and the virus would thereby constitute a neutralisation escape mutant. The importance of the Lys¹⁴¹ residue has also been demonstrated by the fact that acylation of this amino acid inhibits binding of anti-HBs directed against the *a* determinant [Howard and Allison, 1995]. This mutation has been detected in cases of vaccine-escape in Singapore [Chen *et al.*, 2002], whereas K141E has been identified in isolates from The Gambia, West Africa [Karthigesu *et al.*, 1994].

Another clone displayed a P142L amino acid mutation, in which the non-polar pyrrolidine side chain of Pro¹⁴² was replaced by the non-polar aliphatic hydrocarbon side chain of Leu¹⁴². It has been proven that Pro¹⁴² is essential for the display of full antigenicity of the *a* determinant, because the tertiary structure of the antigenic second loop is dependent upon the Pro¹⁴²-induced β -turn between the disulphide bridges [Ashton-Rickardt and Murray, 1989]. Such a turn may be impeded by the bulky side chain of Leu¹⁴², which would

therefore decrease the antigenicity. This variant appears to represent a unique mutation and is likely to constitute a vaccine-escape mutant. Also representing a unique amino acid mutation, T143A occurred within another clone. Because Thr¹⁴³ has a long, polar hydroxylic side chain and is replaced by the short, non-polar methyl group of Ala¹⁴³, the tertiary structure and hydrophilicity of the antigen is likely to be altered, resulting in a reduced antigenicity and immune evasion.

Two clones displayed N146D amino acid mutations within the second loop. Not only would the replacement of the uncharged, polar, amide-bearing side chain of Asn¹⁴⁶ with the negatively charged β -carboxyl group of Asp¹⁴⁶ result in an alteration of the antigenic structure, and thus immunogenicity, but such a mutation additionally results in the loss of the major HBsAg N-glycosylation site at this position. N-linked glycosylation, or oligosaccharide addition to asparagine residues, is an important signal in the correct folding and transport of viral envelope proteins [Wang *et al.*, 1996]. Within the cell, glycoproteins become transiently associated with calnexin, a molecular chaperone localised in the ER membrane during protein maturation [Ou *et al.*, 1993]. Such an association with calnexin requires the presence of N-linked oligosaccharide side chains, which ensures the correct folding of glycoproteins and enhances their maturation [Hammond *et al.*, 1994]. Because it is well established that N-linked glycosylation is also necessary for HBV secretion [Lu *et al.*, 1995], the N146D amino acid mutation, in addition to affecting the structure of the *a* determinant, may result in reduced release of HBsAg from hepatocytes. This specific mutation has been identified previously in an interferon-treated patient [Seddigh-Tonekaboni *et al.*, 2001], but this is the first instance in which the mutant may represent a vaccine-escape virus.

Nine of the 17 serum-derived HBV clones containing MHR-encoding region mutations displayed substitutions outside the α determinant within the MHR. Although such mutations are less significant in terms of vaccine-escape, they may still have an effect on the α determinant's antigenicity and have previously been shown to be able to escape neutralisation by α determinant-specific antibodies [Oon *et al.*, 1999]. Five clones had variations at amino acid 102: one displayed G102A, which is unlikely to cause structural changes because of the similarity of the residue side chains; and four clones had G102S, where the short, non-polar side chain of Gly¹⁰² was replaced by the longer, polar hydroxyl side chain of Ser¹⁰², which could potentially alter the protein structure at that point. Another clone had the amino acid variation M103I within the MHR. The substitution of Met¹⁰³, possessing a non-polar, thiol ether side chain, by Ile¹⁰³, which has a non-polar, aliphatic hydrocarbon, may have a potential effect on the protein, although it is unlikely. Similarly, in a clone displaying V106A, the amino acid side chains have similar properties, which probably negates the effect of the substitution.

In addition to possessing the N146D amino acid mutation, one clone had an S117G substitution outside the α determinant. Because Ser¹¹⁷, an uncharged, hydrophilic residue, was replaced by Gly¹¹⁷, a smaller hydrophobic residue, a consequential change in protein structure and decrease in antigenicity is plausible. Oon *et al.* (1999) identified vaccine-escape mutants with substitutions at residues 116 and 118. Therefore, a change at amino acid 117 may similarly cause immunological escape of the virus.

Displaying a mutation immediately adjacent to the second loop of the α determinant, another clone showed a T148A amino acid variation. Substituting the polar, hydroxylic side chain of Thr¹⁴⁸ with the small, non-polar side chain of Ala¹⁴⁸ would be likely to have

an effect on the tertiary structure of the *a* antigen, because of the residue's close proximity to Cys¹⁴⁷ within the second loop. Such structural change may consequently result in reduced antigenicity.

The amplified surface gene of the patient's liver tissue-extracted HBV was cloned and 57 clones were sequenced within the S gene (nt 256-796). Of these, only two displayed unique or uncommon non-synonymous mutations within the region of the gene encoding the MHR of the HBsAg, when compared to the corresponding sequences of various HBV isolates obtained from GenBank.

The first clone displayed a M103I amino acid mutation outside the *a* determinant, as already described, as well as a C139Y mutation within the highly conserved second loop of the *a* determinant. Similar to the important effect of the C139R mutation on the *a* determinant identified in the serum clone, the substitution of Cys¹³⁹, with a polar thiol side chain, by Tyr¹³⁹, having a larger, polar phenol group, would destroy the disulphide bond maintaining the structural integrity of the second loop, thereby reducing both the antigenicity of the epitopes and the secretion of HBsAg. This mutation has been observed as causing diagnostic assay detection failure [Alexopoulou *et al.*, 2004], but is novel with regard to vaccine-escape.

The second liver-derived HBV clone also had a mutation within the highly conserved second loop – D144N. The substitution of Asp¹⁴⁴, which has a negatively charged, polar β -carboxyl group, with its uncharged amide, Asn¹⁴⁴, would not only be likely to alter the structure and hence the antigenicity of the epitope, but also introduces an additional N-glycosylation site within the HBsAg. Despite the finding of pepscan analyses with murine

anti-KPTDG peptide (aa 141-145) antisera, which concluded that Asp¹⁴⁴ was not as critical for antibody binding as the remaining residues [Steward *et al.*, 1993], the vaccine-escape mutant D144A has been reported from several countries and has been shown to have reduced antibody binding activity [Ni *et al.*, 1995; Ngui *et al.*, 1997]. The D144N mutant identified in this study could therefore represent a novel vaccine-escape mutant.

Since the S gene overlaps the P gene, which is in the +1 reading frame in relation to S, it is conceivable that mutations occurring in the S gene, and causing variation in the *a* determinant of HBsAg, could also produce mutations within the overlapping fingers subdomain of the polymerase enzyme [Torresi *et al.*, 2002]. Although G145R (rt W153Q), D144E (rt G153E), and M133L (rt Y141S) are known to reduce HBV replication [Torresi *et al.*, 2002], this region of the P gene is poorly conserved among mammalian strains of HBV and it is therefore likely that the S gene mutations described in this study had little adverse effects on viral replication. Further studies involving determination of each mutant's replication-competence would be necessary to verify this.

In humans, HBV exists as a heterogeneous population, or quasispecies, consisting of both wild-type and mutant viruses. In cases of vaccine-escape, the selective pressure of vaccine-induced antibodies usually causes the wild-type viruses to be eradicated, thereby allowing the escape mutants to gain dominance and cause infection. In this study, however, there was an unusual dominance of wild-type strains over *a* determinant mutants within both the serum and liver tissue.

Considering that the patient had a protective anti-HBs titre of 17.82 mIU/ml upon infection, it may be presumed that the development of wild-type infection was not the

result of vaccination failure in the host, but rather of vaccine-escape. As such, the reason for the existence of the mutant strains as minor species is uncertain. One possibility is that, upon infection of the host with HBV quasispecies via a needle-stick injury, one or more of the *a* determinant mutants described was able to escape the host immune response and become dominant as the wild-type was simultaneously eradicated. Following infection of the liver by the mutants and the subsequent development of chronic hepatitis, the majority of the viruses could have reverted back to the wild-type strain, as it may have offered greater survival advantages over the mutant strains in terms of replication or infectivity. Such viral strain reversion has been described in liver transplant recipients following the withdrawal of immune pressure by HBV immunoprophylaxis [Ghany *et al.*, 1998].

Alternatively, the presence of the mutant strains as minor species in the serum could be explained by the occurrence of mutants with secretion-defective phenotypes – such as C138R, C139R, and N146D – that are consequently present as dominant species within the liver. However, no such mutants were detected in the liver, so this possibility is unlikely.

Although the possibility cannot be excluded that the mutations detected in the cloned amplicons were the result of PCR artefacts, there were a number of observations that argue against such an interpretation and that support the conviction that these mutations were authentic. Firstly, the mutations were only found within the highly conserved immunodominant B-cell epitopes of the *a* determinant (aa 124-147), whereas no mutations occurred in the highly conserved hydrophobic helices, II (aa 80-98) and III (aa 169-200), that are embedded in the lipid membrane (*Figure 3.8*). Statistical analysis using the Fisher Exact Test revealed statistically significant differences ($p < 0.01$) between the number of clones containing mutations within the *a* determinant and the number containing mutations

in helices I and II. Such results prove that the mutations did not occur randomly, as would have been expected had they been errors incurred during PCR. Secondly, as already described, high fidelity polymerases were used in all experiments in order to minimise the misincorporation of nucleotides into the extending amplicons.

Further studies involving determination of the antigenicity and binding ability of all of these mutant proteins to α determinant-specific monoclonal antibodies would be necessary to verify these hypotheses of vaccine-escape. The transmissibility of the mutant viruses would determine their significance.

Chapter 5: CONCLUSION

This case of breakthrough HBV infection that has occurred in South Africa was likely to have been caused by vaccine-escape mutants of the virus, which escaped neutralisation by vaccine-induced antibodies. The complete genome of HBV isolated from the patient's serum and obtained by direct sequencing, was 3 215 bp in length, displayed a wild-type S gene, and lacked the six nucleotide insert between nt 2 353 and 2 360 that is present in all genotype A isolates. Cloning and sequencing of isolates from the patient's serum and liver revealed a subgenotype A1, predominantly wild-type quasispecies, with a small minority of isolates displaying mutations within the region of the surface gene encoding the antigenic *a* determinant of the HBsAg. Of the amino acid mutations identified, those occurring in the highly conserved second loop of the *a* determinant (C138R, C139R, K141R, P142L, T143A, N146D, and T148A in the clones from the serum, and C139Y and D144N in the clones from the liver), were most likely to have caused the vaccine-escape. The predominance of the wild-type over mutant HBV strains may have been the result of reversion of the latter to the former strain following the establishment of infection. However, more extensive research is required to determine this possibility. Before conclusions concerning vaccine strategies can be deduced from this study, the infectivity and pathogenicity of these mutants will have to be determined. Finally, despite the HBV vaccine being highly effective within the framework of the South African EPI, enhanced surveillance to detect the emergence of vaccine-escape mutants should be a high priority in evaluating the effectiveness of current immunisation strategies.

Chapter 6: REFERENCES

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Appendix A: COMPLETE PATIENT INFORMATION

Table A1 Patient information including ALT and AST levels, and HBV antigen and antibody status

Ethnicity	Caucasian
Gender	Male
Age (Years)	55
Time of Vaccinations	Late 1980s (<i>Intradermally</i>) 1993 (<i>Intramuscular booster</i>) 1999 (<i>Second intramuscular booster following needle-stick injury</i>)
Time of Presentation	June 1999 (<i>Needle-stick injury</i>)
Anti-HBs Titre (mIU/ml)	17.82 (<i>June 1999</i>)
Antigen Status	HBsAg +ve (<i>January 2002</i>)
Anti-HBc Status	IgM +ve (<i>January 2002</i>) IgG +ve (<i>Late 2002</i>)
ALT Level (mIU/ml)	505 (<i>January 2002</i>)
AST Level (mIU/ml)	285 (<i>January 2002</i>)

Appendix B: ETHICS APPROVAL AND PATIENT'S INFORMED CONSENT

UNIVERSITY OF THE WITWATERSTRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

R14/49 Crowther

CLEARANCE CERTIFICATE

PROTOCOL NUMBER M040818

PROJECT

Molecular Characterisation of Hepatitis B
Virus Vaccine Escape Mutants in
South Africa

INVESTIGATORS

Ms P Crowther

DEPARTMENT

School of Clinical Medicine

DATE CONSIDERED

04.08.27

DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application

DATE 04.08.30

CHAIRPERSON.....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

Cc: Supervisor: Dr A Kramvis

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Tel: 2972555
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Orapa
Botswana

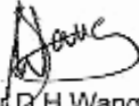
1.03.2004

Re: Consent to use by tissues in research

I hereby authorize Prof. M. C. Kew and any of his collaborators to use my serum, liver biopsy specimens and any other tissues in any research they choose to do. They may also use results or data in whatever manner they deem fit.

I am fully aware of the consequences of this decision and have discussed this decision with medical colleagues

Yours faithfully


1.03.2004
Dr. D. H. Wang.
Chief Medical Officer
Orapa Mine Hospital

Appendix C: SOLUTIONS AND RECIPES

Agarose Gel (0.8%) – Crystal Violet

- * 0.6 g Molecular grade agarose (Whitehead Scientific)
- * 75 ml 1X TAE buffer
- Boil in microwave until all agarose is dissolved;
- Cool to ~ 55°C;
- Add 45 μ l of 2mg/ml crystal violet and mix;
- Pour into clean gel tray with well-comb and allow at least 20 minutes to set.

Agarose Gel (1%) – Ethidium Bromide

- * 1.5 g Molecular grade agarose (Whitehead Scientific)
- * 150 ml 1X TBE buffer
- Boil in microwave until all agarose is dissolved;
- Cool to ~ 55°C;
- Add 15 μ l ethidium bromide and mix;
- Pour into clean gel tray with well-comb and allow at least 30 minutes to set.

Agarose Gel: Composite (3%) – Ethidium Bromide

- * 6 g NuSieve GTG agarose (Cambrex)
- * 3 g Molecular grade agarose (Whitehead Scientific)
- * 300 ml 1X TBE buffer
- Boil in microwave until all agarose is dissolved;
- Cool to ~ 55°C;
- Add 30 μ l ethidium bromide and mix;
- Pour into clean gel tray with well-comb and allow at least 30 minutes to set.

Bromophenol Blue Loading Dye

- * 0.02 g Bromophenol blue (Saarchem)
- * 4 ml 0.1 M EDTA (Saarchem)
- * 10 ml Glycerol (Saarchem)
- Make up to 20 ml with distilled water and mix;
- Aliquot into 1.5 ml microcentrifuge tubes;
- Store at 4°C.

Ethidium Bromide Stock (10mg/ml)

- * 0.1 g Ethidium bromide (Sigma)
- * 10 ml Best quality water
- Mix thoroughly and store in a dark bottle at 4°C.

Ethylenediamine Tetraacetic Acid (EDTA) Stock (1M)

- * 37.22g Na₂EDTA.2H₂O (Saarchem)
- * 80 ml Distilled water
- Adjust to pH 8.0 with NaOH pellets;
- Make up to 100 ml with distilled water;
- Autoclave;
- Store at room temperature.

Luria-Bertani (LB) Agar Plates with Kanamycin

- * 5 g Tryptone (Pronadisa)
- * 2.5 g Yeast extract (Pronadisa)
- * 5 g Sodium chloride (Saarchem)
- Make up to 500 ml with distilled water and mix thoroughly;
- Add 10 g Bacteriological agar (Whitehead Scientific);
- Autoclave;
- Cool to ~ 55°C;

- Add 500 μ l of 50 μ g/ml kanamycin antibiotic;
- Pour into sterile 10 cm plates;
- Allow to harden, then invert and store at 4°C in the dark.

Luria-Bertani (LB) Medium

- * 5 g Tryptone (Pronadisa)
- * 2.5 g Yeast extract (Pronadisa)
- * 5 g Sodium chloride (Saarchem)
- Make up to 500 ml with distilled water;
- Autoclave;
- Store at 4°C.

TAE Buffer (10X)

- * 24.2 g Tris base (Roche)
- * 5.71 ml Glacial acetic acid (Saarchem)
- * 10 ml 0.5 M EDTA
- Make up to 500 ml with distilled water;
- Autoclave;
- Store at room temperature.

TBE Buffer (10X)

- * 108 g Tris base (Roche)
- * 55 g Boric acid (Sigma)
- * 7.44 g Na₂EDTA.2H₂O (Saarchem)
- Make up to 1 L with distilled water;
- Autoclave;
- Store at room temperature.