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A RETROSPECTIVE STUDY CHARACTERIZING THE COMPLETE S OPEN READING FRAME OF HEPATITIS B VIRUS FROM BLACK CHILDREN WITH MEMBRANOUS NEPHROPATHY TREATED WITH INTERFERON ALPHA-2b

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

Johannesburg, 2006

DECLARATION

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

……

…….. day of …… 2006

ABSTRACT

In sub-Saharan Africa a causal relationship has been established between hepatitis B virus (HBV) infection and membranous nephropathy (MN), especially in Black children. The most common method of treatment is interferon therapy, which is however, only effective in 30-40% of patients. The reason for this is unclear. The objective of this pilot study was to determine whether mutations in the complete surface gene of HBV isolated from Black children with HBV-associated MN before, during and after treatment with interferon, had any effect on treatment response and *vice versa*. HBV DNA was extracted from the serum of a responder, reverter and non-responder patient before, during (4 and 16 weeks) and after (40 weeks) IFN treatment. The preS1/preS2/S region was amplified and cloned, and the clones sequenced. Sequence analyses revealed the preS2 region to be the most variable in the reverter and non-responder and HBsAg was the most variable in the non-responder. Phylogenetic analysis showed that the viral population dynamics between the responder strains and the reverter/non-responder strains differed as a result of various mutations found within the surface gene. Thus the presence of mutations in preS2 and HBsAg of the non-responding patients may carry predictive markers for non-response but further investigation would be needed to conclusively prove this.

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LIST OF ABBREVIATIONS

3'A	3' deoxyadenosine
aa	Amino acid
ALT	Alanine aminotransferase
Anti-HBe	Hepatitis B e antibodies
BCP	Basic core promoter
bp	Base pair(s)
BQW	Best quality water
cccDNA	Covalently closed circular DNA
CHB	Chronic Hepatitis B
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR	Direct repeat
EPI	Expanded Programme on Immunisation
ER	Endoplasmic reticulum
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBVMN	Hepatitis B virus-associated membranous nephropathy
HBx	Hepatitis B x antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Hsp	Heat shock protein
IFN	Interferon
ISG	Interferon-stimulated genes
kb	Kilo base(s)
kDa	KiloDalton(s)
LB	Luria-Bertani
MEIA	Microparticle enzyme immunoassay
MHC	Major histocompatibility complex
MHR	Major hydrophilic region
min	Minute(s)
MN	Membranous nephropathy
mRNA	Messenger ribonucleic acid
NON-RESP	Non-responder
nt	Nucleotide(s)
ORF	Open reading frame
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA

rcDNA		Relaxed circular DNA
RESP		Responder
REV		Reverter
RFLP		Restriction fragment length polymorphism
RNA		Ribonucleic acid
RNase		Ribonuclease
rpm		Revolutions per minute
RT-PCR		reverse trancriptase polymerase chain reaction
S		Surface
sec		Second(s)
SNP		Single nucleotide polymorphism
Surface proteins	–L	Large
	M	Medium
	S	Small
T		Deoxythymidines
TP		Terminal protein
V		Volts
ε		Epsilon

Chapter 1: INTRODUCTION

Hepatitis B virus (HBV) is an important cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. In addition, a causal relationship has been established between HBV and membranous nephropathy (MN), a kidney disorder that may occur, especially in children (Bhimma *et al.*, 1998). With an estimated 385 million carriers of HBV world-wide, and a mortality rate of 1.2 million deaths per year as a result of acute or chronic HBV infection (King *et al.*, 2002), HBV remains a major public health problem. In Africa, with a population of approximately 470 million people and where HBV is hyperendemic, as many as 98% of the population are infected with HBV at some point in their lives (Kew, 1996). Equally impressive, is the fact that more than 230 000 Africans will die each year from HBV-induced diseases (Kew, 1992).

Even though an effective and safe vaccine for the prevention of HBV infection exists, the treatment of those already living with chronic HBV infection is unsatisfactory. Interferon-alpha (IFNα) was the first approved agent for the treatment of chronic HBV infection (Lai *et al.*, 2002a). Its efficacy is however, limited. In a previous study, IFNα was used to treat nineteen Black children with HBV-associated membranous nephropathy. At 40 weeks of follow-up, ten patients (52.6%) had cleared HBeAg (responders), four (21.1%) had cleared HBeAg during treatment but then reverted to being HBeAg-positive (reverters), and five patients (31.6%) failed to clear HBeAg (non-responders). None cleared HBsAg. The aim of the present study was to determine

whether differences in the genomic structure of HBV isolates from IFNα treated patients could account for the variation in response to treatment.

1.1 HEPATITIS B VIRUS

1.1.1 Classification

HBV belongs to the family of enveloped DNA viruses, the *Hepadnaviridae,* which share a similar structural and genomic organization (Scaglioni *et al*., 1996). The members of this family that infect mammals are grouped in the genus *Orthohepadnavirus,* whereas those that infect birds belong to the genus *Avihepadnavirus* (Seeger and Mason, 2000). All hepadnaviruses contain a partially double-stranded DNA genome, but replicate via an RNA intermediate, the pregenome, which encodes an error-prone polymerase enzyme with both DNA polymerase and reverse transcriptase activity (MacDonald *et al.*, 2000).

Hepadnaviruses have a narrow host range, with HBV being able to infect humans and chimpanzees only (Lai, 2002b). The primary target of infection with HBV and other human hepatitis viruses is the liver, with transmission being via the parenteral route.

1.1.2 Epidemiology

HBV infection occurs throughout the world but in widely differing frequencies. Socioeconomic factors, the proportion of the population with risky lifestyles, the current prevalence of HBV, the availability of vaccination programs and compliance to hygienic measures, all contribute to a country's epidemiological status (Grob, 1995). The most common routes of HBV transmission include needle sticks (accidental injuries and drug use), sexual contact, blood transfusions and mothers to newborns (Grob, 1995).

The world can generally be divided into low (<2%), intermediate (2-8%) and high (>8) endemicity areas (*Figure 1.1*) (McMahon, 2005), but this is changing over time following the introduction of vaccination programs. There may also be substantial epidemiological differences between countries in the same continent. In general, HBV is highly endemic in developing countries with large populations and most infections in highly endemic areas occur during infancy or early childhood (Hou *et al.,* 2005). Most of Asia, the South Pacific Island region and the Arctic/sub-Arctic are high endemicity areas (Hou *et al.,* 2005; McMahon, 2005). Amongst the South-East Asian areas, China, Taiwan, Korea and Thailand are classified as high endemicity areas, with a 7-20% prevalence of hepatitis B surface antigen (HBsAg) (André, 2000). HBV is highly endemic in most of West, East, Central and sub-Saharan Africa with chronic infection rates of 7-26%, although areas like Tunisia, Morocco and Zambia fall into the intermediate category (André, 2000). Egypt, Jordan, Oman, Palestine, Yemen and Saudi Arabia are also high endemicity areas (André, 2000).

In areas of moderate endemicity, transmission is usually mixed and includes infant, early childhood and adult transmission (Hou *et al.*, 2005). The Mediterranean, Southern and Eastern Europe, North Africa, the Middle East, India and parts of South America fall into the intermediate endemicity category (McMahon, 2005) as do Cyprus, Iraq, India, the Philippines and the United Arab Emirates (André, 2000).

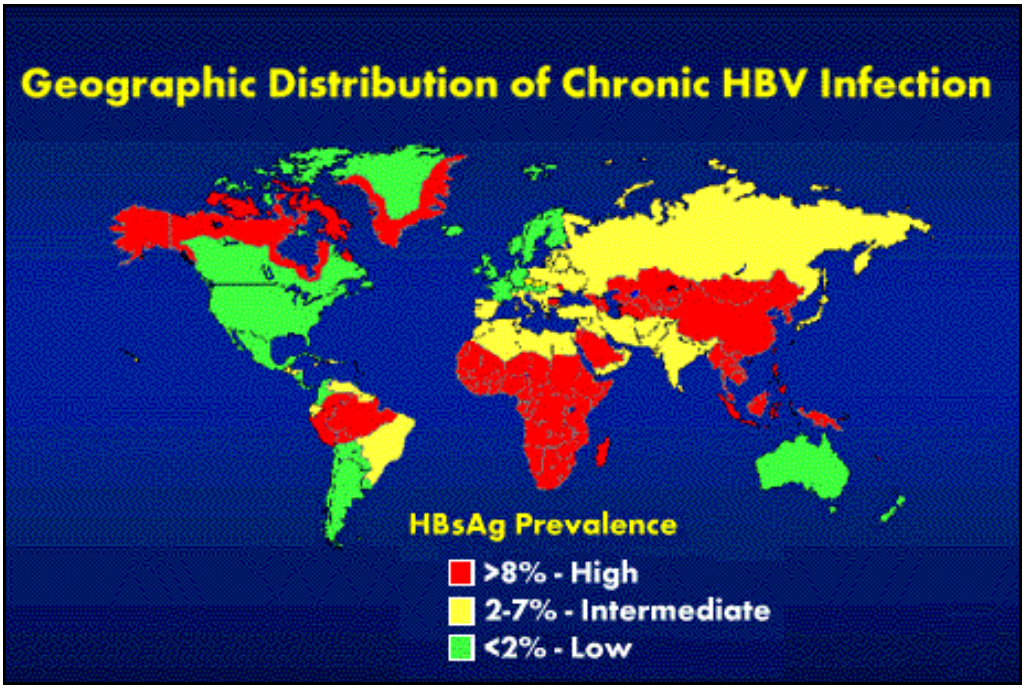


Figure 1.1: Worldwide distribution of chronic HBV infection showing the high, intermediate and low prevalence areas (www.safetyline.wa.gov.au/institute/level2/course20/lecture81/l81_02.asp).

Most areas in the Middle East, Bahrain, Iran, Israel and Kuwait are low endemic areas because of mass vaccination programs, which have begun to control the spread of HBV by decreasing HBsAg carrier rates to under 2% (André, 2000). In developed areas such as the United States of America and Northern and Western Europe, HBV infection rates are low (Hou *et al.*, 2005), whilst Japan, Pakistan, Bangladesh and Sri Lanka are also seen as low endemic areas with a HBsAg prevalence of 0.2-1.9% (André, 2000). Transmission of HBV infection in low endemic areas are generally as a result of well-defined high risk groups such as drug-users, homosexuals, blood transfusion patients and health care workers (Hou *et al.*, 2005)

1.1.3 Transmission

HBV can be transmitted horizontally by blood, blood products and body fluids such as saliva, sweat, semen, breast milk, urine and faeces (Prescott *et al*., 1999), with blood products and semen carrying a greater risk than other bodily fluids. Chronic HBV infection is very common in sub-Saharan Africa where the high carrier rate of the virus is largely established in early childhood (1-5 years of age), mainly by horizontal transmission of the virus via contact with siblings and unrelated playmates as well as other family members (Kew, 1996). These are the most common routes of transmission amongst children of this age (Tsebe *et al*., 2001). Bites from mosquitoes and bed bugs, as well as scarification of the skin by witch doctors, might play a lesser role in the horizontal spread of HBV in sub-Saharan Africa (Kew, 1996).

HBV infected mothers may also pass the virus from their blood onto the fetus, through the placenta, more commonly termed vertical transmission. It has been shown that infants have a greater than 90% chance of becoming HBV carriers if their mothers are hepatitis B e antigen (HBeAg) positive (Hino *et al*., 2001). Vertical transmission however, even from highly infectious mothers, appears to be rare in Africa. When a HBV carrier mother infects the child during or shortly after delivery, it is termed peri-natal transmission. Transmission via this route is uncommon in Africa but occurs frequently in China and the Far East.

1.1.4 Biology

The HBV virion or Dane particle is made up of an outer shell, consisting of envelope proteins and a small amount of lipid, and an inner nucleocapsid (Scaglioni *et al*., 1996; Seeger and Mason, 2000). The virion has a diameter of 27 – 42nm. Within the lumen of the nucleocapsid (core), is contained the partially double-stranded relaxed circular DNA (rcDNA), covalently bound to the viral polymerase (*Figure 1.2*) (Kann, 2002). The negative strand of the HBV genome is approximately 3.2 kb in length and consists of four open reading frames encoding viral proteins and *cis*-acting elements needed for regulating HBV gene expression and replication (Arbuthnot *et al*., 2000). The circular structure of the genome is maintained by the positive strand of variable length which cohesively binds to the 5' and 3' ends of the negative strand (Arbuthnot *et al*., 2000).

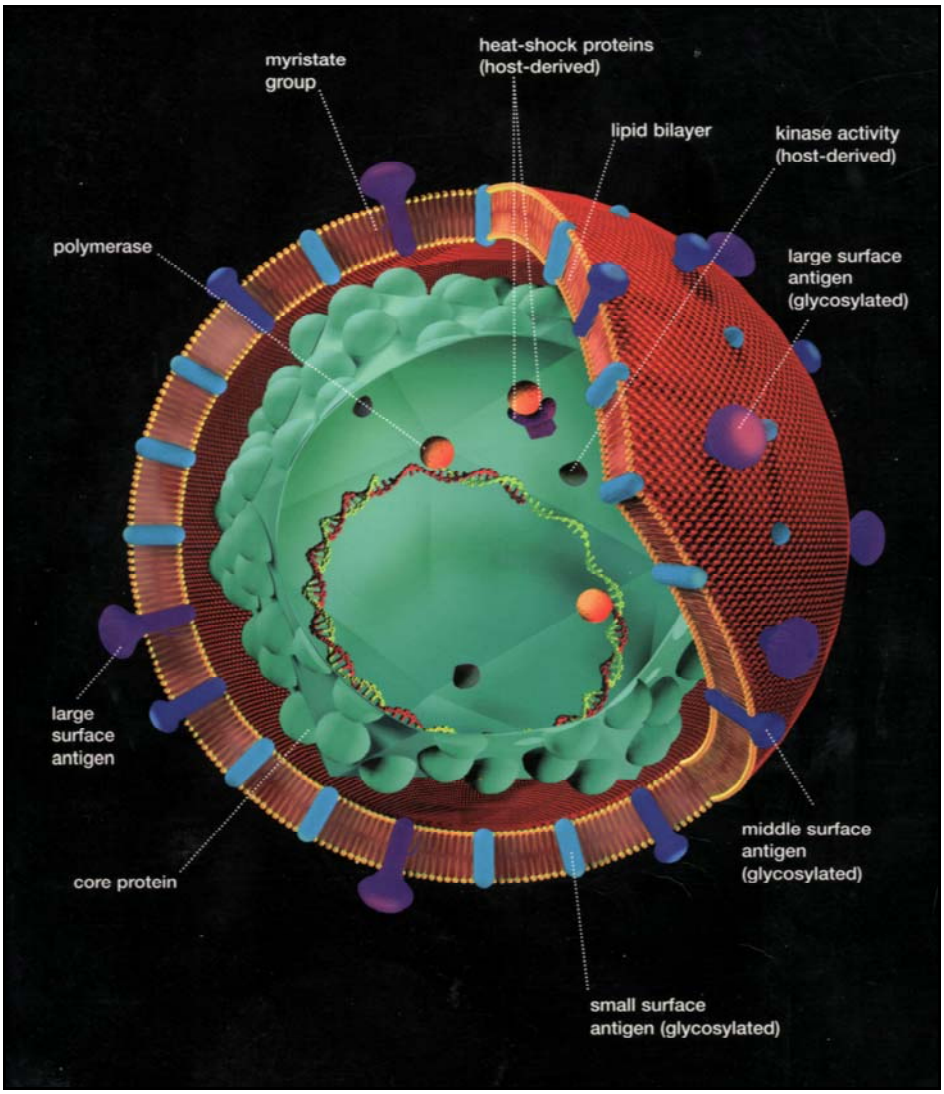


Figure 1.2: The morphology of a hepatitis B virion demonstrating the double layered shell consisting of the small, middle and large glycosylated surface proteins as well as the lipid bilayer; the core made up of core proteins; the polymerase protein; the enzymatic proteins and the partially double stranded DNA genome. Heat shock proteins are found within the envelope shell (Kann, 2002).

Besides HBV virions, the serum of infected individuals also contains an excess of subviral particles: spherical ~22nm particles and filamentous particles that vary in length (*Figure 1.3*) (Prescott *et al*., 1999). These spheres and filamentous particles consist of viral surface proteins and host-derived lipid components (Kann, 2002) only, and unlike the Dane particle, are non-infectious (Prescott *et al*., 1999). During infection, circulating viral particles may have a concentration ranging from 10^3 to 10^9 viral particles per millimeter of serum, whilst subviral particles may range anywhere from between 10^6 and 10^{14} particles per millimeter (Scaglioni *et al*., 1996). Although these subviral particles lack DNA or RNA and are therefore not infectious, they are nevertheless highly immunogenic.

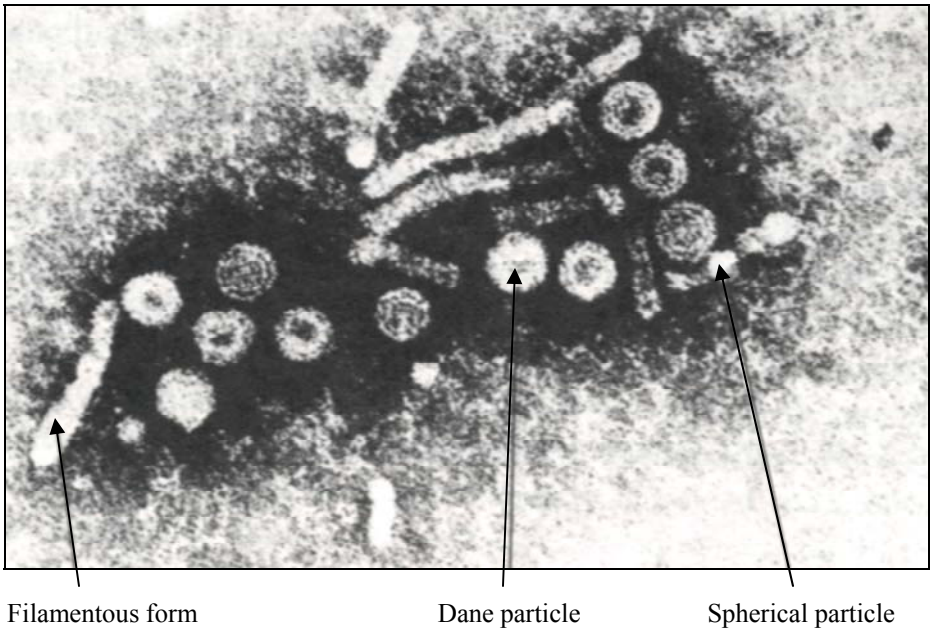


Figure 1.3: An electron micrograph (x210,000) of hepatitis B virus in serum showing the three distinct subviral particles; the spherical and filamentous particles consisting of only viral surface proteins and host-derived lipid components and the complete infectious virion or Dane particle (Prescott *et al*., 1999).

1.1.5 Lifecycle

1.1.5.1 Entry into Hepatocytes

Entry of the virus into the host cells is mediated by the initial attachment of HBV to an as yet unidentified receptor, followed by receptor-mediated endocytosis into the hepatocytes (*Figure 1.5*) (Scaglioni *et al*., 1996). Once inside the host cell, the viral envelope is removed by host factors and the core protein conveys the viral genome to the nucleus via a nuclear localization signal contained within the nucleocapsid (Yeh *et al.,* 1990; Scaglioni *et al*., 1996). Before entering the nucleus, the nucleocapsid is disassembled on the cytoplasmic side of the nuclear membrane, thus allowing the viral DNA to enter the nucleus and initiate replication (Scaglioni *et al*., 1996).

1.1.5.2 Formation of Covalently Closed Circular DNA (cccDNA)

The first step in HBV replication is the conversion of the HBV genome from rcDNA to a double-stranded covalently closed circular DNA (cccDNA) (*Figure 1.5*). The cccDNA acts as the template for synthesis of all viral transcripts involved in protein production and replication (Doo and Liang, 2001; Arbuthnot *et al*., 2000). A 3.5 kb mRNA or pregenomic RNA (pgRNA) encodes the core and polymerase proteins, the 2.4 kb and 2.1 kb transcripts encode the surface envelope proteins, whilst a 0.7 kb transcript encodes the X-protein (*Figure 1.4*) (Doo and Liang, 2001; Ganem and Schneider, 2001). Transcription of these four proteins is initiated by different gene promoters; the enhancer II/basal core, large surface antigen, major surface antigen and enhancer I/X, respectively.

Transcripts terminate at a single polyadenylation site on the cccDNA (Arbuthnot *et al*., 2000; Ganem and Schneider, 2001). The pgRNA is longer than the full genome length of HBV and the 5' and 3' ends contain a terminal redundancy region, which act as the *cis*-acting motifs, namely DR1, a direct repeat of 12 nucleotides, and epsilon (ε), a secondary stem-loop structure (Scaglioni *et al*., 1996).

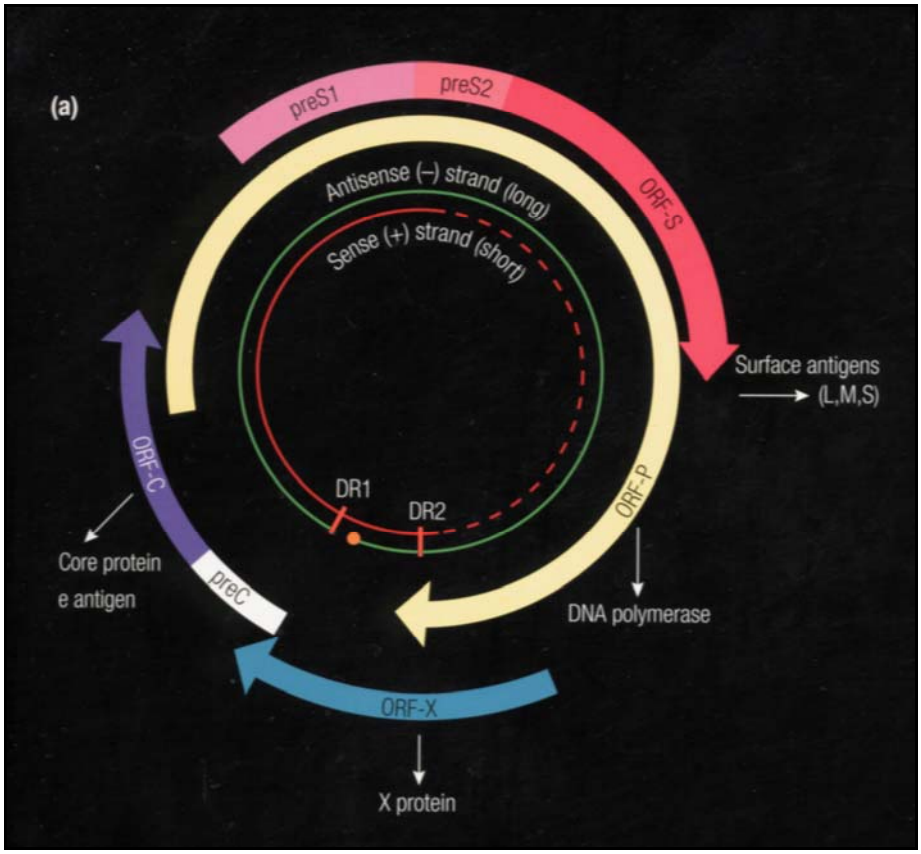


Figure 1.4: The circular structure of the HBV genome showing the plus (+) and minus (-) sense strands. The arrow bars indicate the four overlapping open reading frames of HBV and their corresponding protein products. DR1 and DR2 are also illustrated on the 5' and 3' ends (Kann, 2002).

For reverse transcription and encapsidation of the pgRNA to occur, the viral polymerase has to bind to the 5' ε on its own RNA template, via 3 host chaperone proteins. p23 and heat shock protein 70 (Hsp70) aid the binding of the polymerase to ε, whilst Hsp60 is involved in the activation of the viral polymerase (Park and Jung, 2001). This interaction further leads to complex formation with the core proteins, to form nucleocapsids (Doo and Liang, 2001). A second function of this interaction is the initiation of reverse transcription of the pgRNA to yield a negative DNA strand. This only occurs when polymerase binds to ε and acts as a primer to initiate the reverse transcription of a 3 nucleotide long oligonucleotide that will covalently link to the polymerase (Scaglioni *et al*., 1996; Doo and Liang, 2001). Translocation and annealing of the polymerase-primer complex to the complementary 3' DR1 end sequence of the pgRNA, leads to the synthesis of a negative stranded DNA.

DNA strand synthesis occurs within the nucleocapsid, whilst the polymerase derived ribonuclease (RNase) H activity degrades all but the last 15-18 nucleotides at the 5' end of the pgRNA (Scaglioni *et al*., 1996; Doo and Liang, 2001). It is this undegraded RNA molecule that serves as the template for positive DNA strand synthesis when it transfers to DR2, the direct repeat complementary to DR1 but located downstream from the 5' end copy on the negative DNA strand. Circularization of the HBV genome occurs once positive strand synthesis has extended to the end of the negative strand. Binding of the core to the N-terminal domains of the envelope proteins, leads to the translocation of the core across the endoplasmic reticular membrane, into the Golgi complex, and ends with

the secretion of mature virions into the bloodstream (*Figure 1.5*) (Seeger and Mason, 2000).

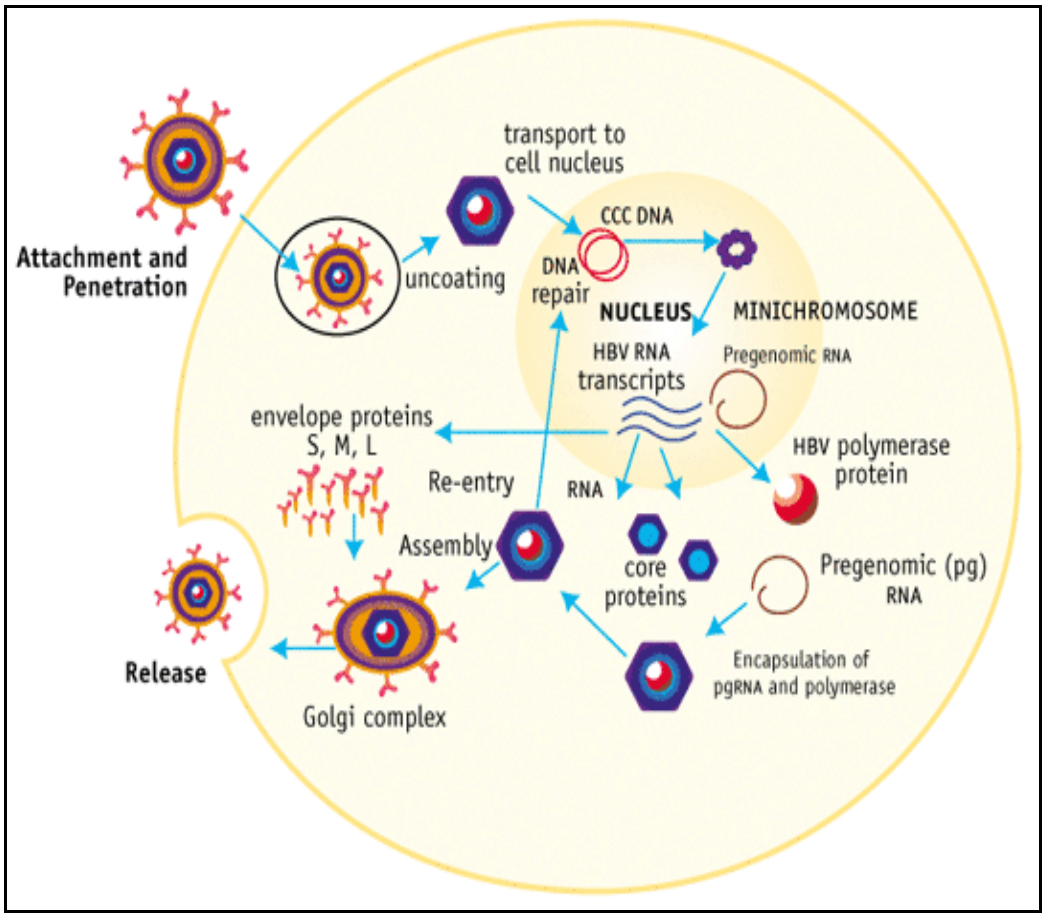


Figure 1.5: Binding of HBV to the surface of the hepatocyte via the envelope proteins, leads to entry into the cell by endocytosis. Once inside, the virus is transported to the nucleus where the HBV genome is converted from rcDNA to cccDNA. Transcription of mRNA and the pgRNA occurs using the cccDNA as the template. The RNAs move to the cytoplasm where they are translated to produce viral proteins. Binding of the core to all three envelope proteins leads to the translocation of the virus across the endoplasmic reticulum and ends with the secretion of mature virions into the bloodstream.
(www.prn.org/prn_nb_cntnt/vol6/num1/img_pgs/locarnini.fig1.htm)

1.1.6 Gene expression

1.1.6.1 Precore/Core ORF

The precore/core open reading frame (ORF) contains two start codons coding for two overlapping proteins (Ganem, 1991). If translation begins at the second AUG start codon, a 21.5 kDa core protein is synthesized, which can be either 183, 185 or 195 amino acids in length depending on viral genotype (Kann, 2002; Locarnini *et al*., 2003). The core protein assembles into the nucleocapsid or viral capsid, and is known as the hepatitis B virus core antigen (HBcAg) (Seeger and Mason, 2000). Two distinct functional domains within the core protein can be distinguished. Amino acids 1-144 of the core protein are needed for assembly of the nucleocapsid, while amino acids 150-164 at the arginine-rich C-terminal end of the protein mediate nucleic acid binding, encapsidation of the pgRNA and stabilization of the capsid protein (Kreutz, 2002; Locarnini *et al*., 2003). The remaining amino acids, 165-173, may be involved in DNA replication. Assembly of core particles is mediated via the dimerization of the first 147 amino acids, resulting in the formation of subviral core particles held together by disulphide bonds (Seeger and Mason, 2000; Kann, 2002). The dimerized particles then assemble into an icosahedral shell consisting of T3 (180 core proteins) or T4 (240 core proteins) symmetries and a diameter of 32 or 36nm (Seeger and Mason, 2000). One hundred and twenty 2nm spikes can be visualized on the surface of the icosahedrons and represent immunodominant regions.

Translation from the first AUG, or precore start codon, will generate a precore/core precursor protein. The first 19 amino acids of the precore protein form a signal which mediates the translocation of the protein to the endoplasmic reticulum (ER). Once there, the 19 amino acids are cleaved off by a host cell signal peptidase and a variable number of amino acids are cleaved off from the C-terminal end (Ganem and Schneider, 2001; Locarnini *et al*., 2003). The resulting protein is correctly folded in the ER. It is 15-18 kDa in size and termed the HBeAg. HBeAg is secreted via the Golgi apparatus and is also expressed on the surface of hepatocytes. The HBe protein is involved in the establishment of persistent infection (Locarnini *et al*., 2003) and may also function as an immunoregulator by behaving as a tolerogen in order to inactivate specific T-cell activity and promote chronicity (Chen *et al.*, 2005).

1.1.6.2 Polymerase ORF

The second translation product from the pgRNA is the viral polymerase. The polymerase ORF is the longest and encodes the multifunctional 90 kDa enzyme via an internal initiator codon on the pgRNA (Seeger and Mason, 2000). The multifunctional nature of the 845 amino acid viral polymerase is as a result of the distinct domains on the protein, (Doo and Liang, 2001; Kann, 2002). The N-terminal domain codes for the terminal protein (TP), which is involved in minus-strand DNA synthesis and is followed by a spacer region with no specific function (Kreutz, 2002; Locarnini *et al*., 2003). A third domain codes for the recognition site of the enzyme and is termed the catalytic domain,

while a fourth domain at the C-terminal end encodes RNase H activity (Kreutz, 2002; Locarnini *et al*., 2003).

1.1.6.3 X ORF

The X ORF is highly conserved and encodes a 154 amino acid, 17 kDa protein termed HBx (Locarnini *et al*., 2003). HBx is divided into 6 domains, A-F, the C-terminus of the protein being the transactivating portion, whilst the N-terminal region is involved in repression of HBx transactivation, thereby having a self-regulatory role (Kreutz, 2002).

HBx is believed to be a transcriptional activator, implicated in hepatocarcinogenesis (HCC) by inhibiting the tumor suppressor protein p53, apoptosis and the repair of damaged hepatocyte DNA (Arbuthnot *et al.*, 2000). It also plays an essential, albeit poorly understood role, in the establishment of HBV infection (Kann, 2002).

1.1.6.4 Envelope ORF

Three distinct surface/envelope proteins, large (L), medium (M) and small (S), are produced by HBV to form the envelope of the virus and can be distinguished by three domains (Heerman *et al.*, 1984) (*Table 1.1).*

Table 1.1: The 3 domains of the envelope/surface proteins

Surface proteins	Domains
Large	PreS1, PreS2 and S
Medium	PreS2 and S
Small	S

All three polypeptides are encoded by the same ORF via three in-frame translation initiation codons (AUG's) and a common stop codon (Gerner *et al*., 1998). As a result of this, all three proteins share the same carboxy-terminus but differ at their amino-terminal ends (Chang *et al*., 2004; Raimondo *et al*., 2004). All three of the envelope proteins are glycosylated to different extents, are type II transmembrane proteins, and are stabilized by disulphide bridges formed by cysteine residues (Seeger and Mason, 2000). The preS1 and S proteins are essential for the viral life cycle, but preS2 appears to be dispensable for virion formation, secretion and infectivity (Raimondo *et al*., 2004).

Synthesis of the envelope proteins is regulated by two separate promoters; the preS1-promoter is responsible for transcription of mRNA to be translated into the preS1 or large surface antigen, and the S-promoter that is responsible for transcripts with different 5' ends, which are then translated into the preS2 or middle and S antigen or small protein (Chang *et al*., 2004).

1.1.6.4.1 Small Envelope Protein

Also referred to as HBsAg, this protein is composed of 226 amino acids and is the most abundantly found of all three envelope proteins (*Figure 1.6a*) (Khan *et al*., 2004).

50%-60% of the small envelope protein is folded into α-helices numbered I – V, by 4-5 lengths of hydrophobic amino acids (*Figure 1.6a*) (Kann, 2002). Amino acids 11-29 of helix I, crosses the ER membrane and translocates the upstream sequence into the ER lumen (Kann, 2002). In infected hepatocytes, HBsAg can be found buried in the endoplasmic reticulum membrane, with residues 29-79 forming the major cytoplasmic loop (Khan *et al*., 2004). The region between helix I and II, corresponding to amino acids 80-98, is found in the cytoplasm and therefore has no epitopes (Kann, 2002). Residues 101–168 of helix II, is termed the immunodominant region. This region contains a high number of cysteine residues, which are cross-linked with each other to form a loop configuration, the major antigenic determinant of HBsAg, known as the 'a' determinant (Locarnini *et al*., 2003). The 'a' determinant is common for all four serological subtypes and is defined by a threonine or isoleucine at position 126 of HBsAg (Kramvis *et al.*, 2005). Amino acid substitutions at positions 122 and 160 of HBsAg are used to distinguish between serotypes *d/y* and *w/r* (Okamoto *et al*., 1987a). The 'a' determinant (amino acids 124-147) is clinically the most important because during infection antibodies against all determinants will be produced, but it is only those against the 'a' determinant that will protect against other HBV serological subtypes (Kreutz, 2002). Because of the abundance of cysteine residues in the immunodominant loop, this region

may also be involved in the formation of intramolecular and intermolecular disulphide bonds, which may lead to the important event of oligomerization of HBsAg and thus its secretion (Khan *et al.,* 2004). Downstream of helix II is helix III, passing through the ER, with helices IV and V spanning the ER membrane (Kann, 2002).

1.1.6.4.2 Medium Envelope Protein

The medium envelope protein, or preS2, is composed of the S domain in addition to 55 amino acids on its N-terminus (*Figure 1.6b*) (Locarnini *et al*., 2003; Chang *et al*., 2004). PreS2 is N-glycosylated with a glycan at amino acid 4 and may additionally have an O-glycan at position 37 in HBV genotypes B-F (Kann, 2002). 90% of preS2 molecules are further modified by the acetylation of their N-terminal methionines (Kann, 2002).

The role of the preS2 protein is not well defined, but seems to be unnecessary for virion assembly, secretion and infectivity as demonstrated by *in vitro* and *in vivo* studies (Gerner *et al*., 1998; Raimondo *et al*., 2004). Because of this, the emergence of preS2-defective mutants has become apparent in HBV infected individuals. PreS2 variants can be grouped into two distinct types (Raimondo *et al.,* 2004):

- Variants unable to synthesize the M protein because of mutations at the level of the preS2 start codon,
- Variants that produce shortened M surface proteins because of in-frame deletions.

As preS2 has been shown to bind specifically to fibronectin found in human liver sinusoids, Kreutz *et al* (2002) proposed that, contrary to other studies, it may be involved

in viral attachment to host tissues. Amino acids 3-16 have also been shown to contain a binding site for human serum albumin, thus further hinting to a role in the enhancement of virus and target cell binding (Kann, 2002).

1.1.6.4.3 Large Envelope Protein

The preS1 domain is made up of the entire S and preS2 domains, in addition to an extra 108-119 amino acids on its N-terminus, depending on subtype/genotype (Raimondo *et al*., 2004). The large envelope protein may have different topologies; it can either be located in the ER and therefore exposed to the surface (*Figure 1.6c*), or it can be located in the cytosol and thus localized in the lumen (*Figure 1.6d*) (Kann, 2002). On account of this, preS1 may have two separate roles.

The large envelope protein contains two important epitopes. One is believed to be recognized by antibodies involved in viral clearance, and the other may be a hepatocyte binding region (Kreutz, 2002). It has since been shown by Glebe *et al* (2005), that preS1 is acylated by a myristic acid at glycine 2, which allows for efficient infectivity of the virus to its target cell, and that sequence 2-48 of preS1 mediates this attachment. This is in accordance with a previous study in which myristylation was found to be conserved among all hepadnaviruses, thus suggesting an important role in the viral lifecycle (Grippon, 1995).

PreS1 therefore seems to be essential for attachment of the virus to its target cell. The moderate hydrophobicity of the N-terminal myristoyl acid promotes protein to membrane

interactions that, during the interaction of the preS1 and its receptor, inserts into the membrane and thus strongly enhances this interaction, leading to nucleocapsid entry into the cytoplasm (Glebe *et al*., 2005). The preS1 also has antigenic sites for B and T cells involved in recovery and protection from viral infection (Locarnini *et al*., 2003). When preS1 anchors itself to the ER membrane, it exposes itself in its entirety to the cytosol, and residues 103-124 have thus been implicated in HBV envelopment and secretion (Khan *et al*., 2004).

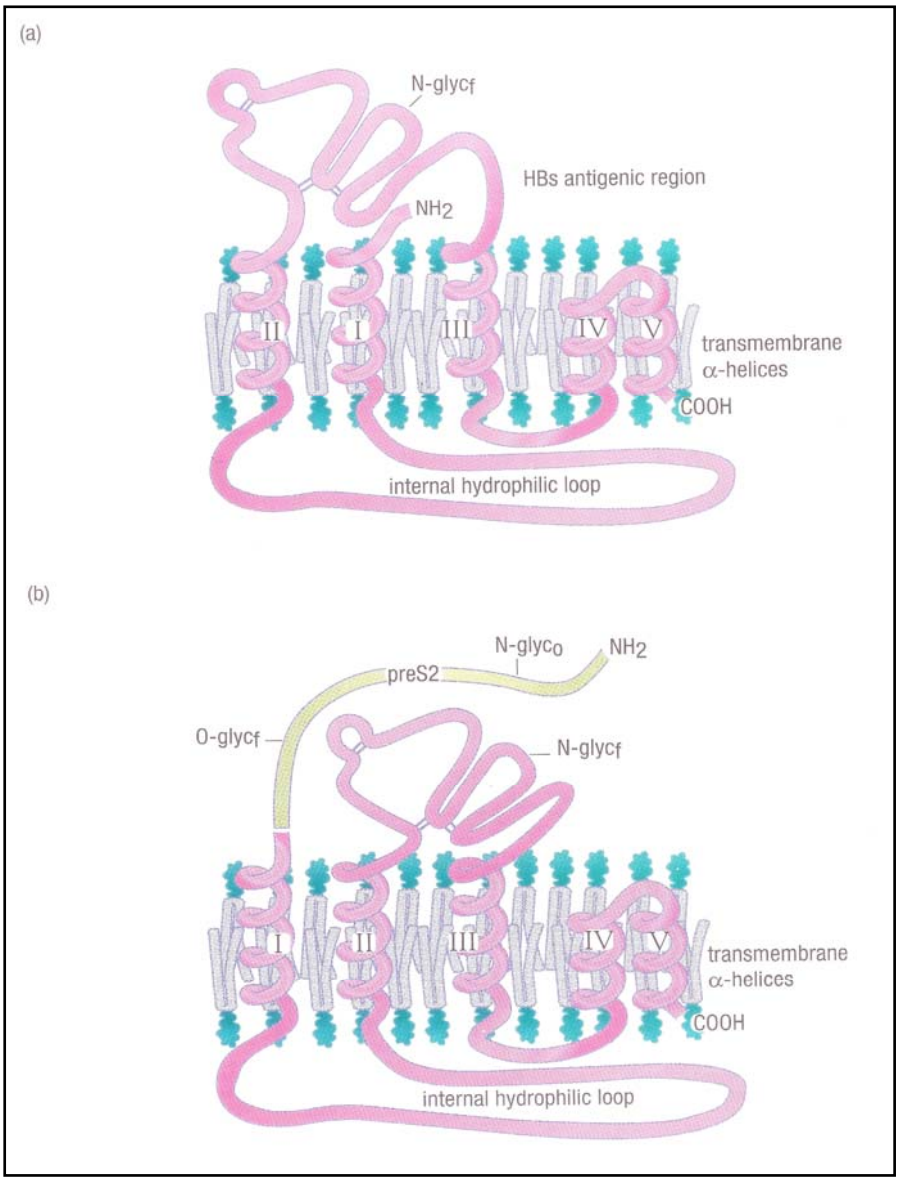


Figure 1.6: Topologies of the 3 surface proteins. (a) Small surface protein (b) middle surface protein. The top of each picture represents the lumen of the ER/preGolgi compartment, whilst the bottom corresponds to the cytosol or interior of the virus. The α-helices are numbered I-V. N- or O-glycosylations are marked N- or O-glyc (Kann, 2002).

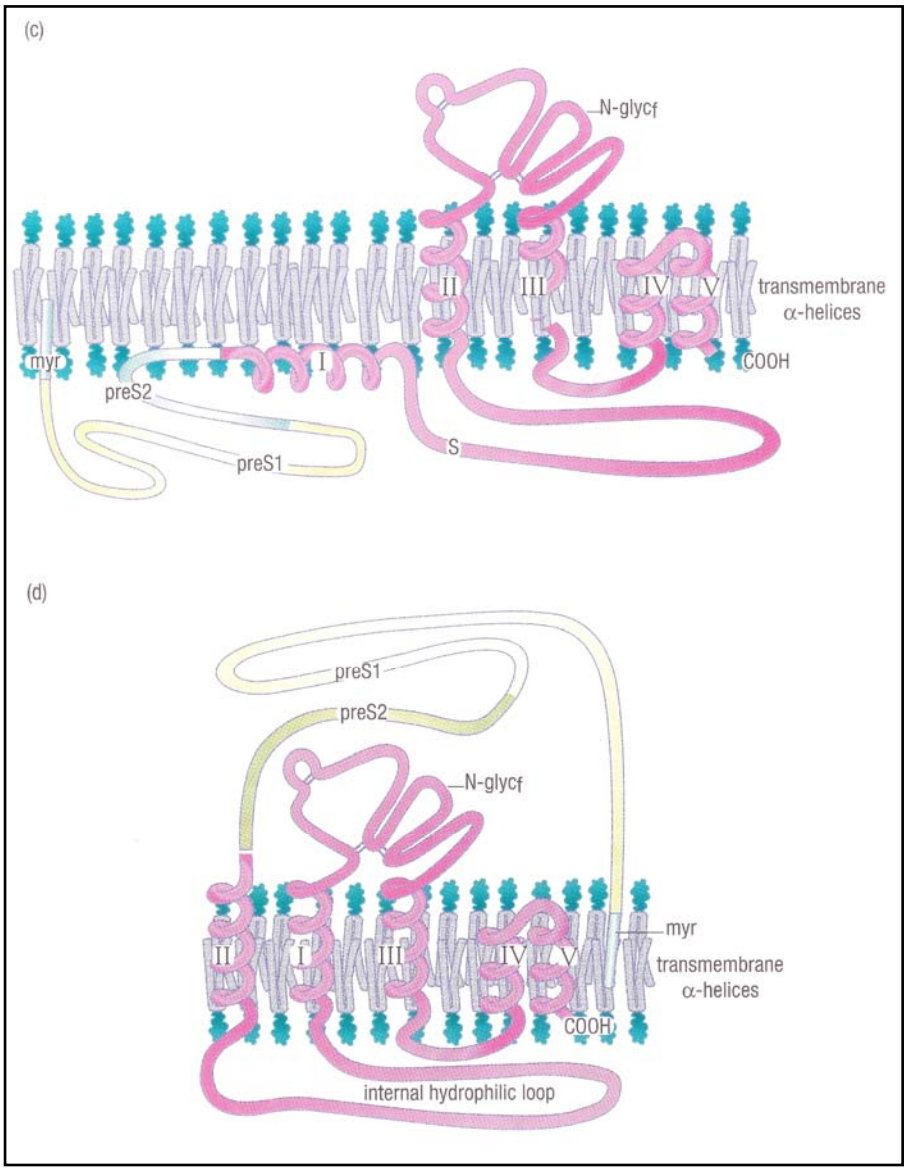


Figure 1.6: Topologies of the 3 surface proteins. (c) Large surface protein with ER localized preS (d) large surface protein with cytoplasmically localized preS. The top of each picture represents the lumen of the ER/preGolgi compartment, whilst the bottom corresponds to the cytosol or interior of the virus. The α-helices are numbered I-V. N- or O- glycosylations are marked N- or O-glyc (Kann, 2002).

1.1.7 HBV Genotypes

A viral genotype refers to the form in which the genomic sequence of a virus that is replication competent has stabilized after a prolonged period of time (Francois *et al*., 2001, Kramvis *et al.*, 2005). HBV polymerase lacks proof-reading activity and the genome may evolve at an estimated error rate of 1.4 - 5 x 10^{-5} nucleotide substitutions/site/year (Okamoto *et al.*, 1987b). As a result of this evolution, genotypes and subgenotypes of HBV have been identified (Kramvis *et al*., 2005). Eight genotypes (A-H) of HBV can be defined by a nucleotide divergence in the region of 8% over the entire HBV genome and >4% over the S region (Norder *et al*., 1992a; Norder *et al*., 1992b). The importance of HBV genotypes has become increasingly significant as their influence on chronic liver disease and response to antiviral therapies has become apparent (Akuta & Kumada, 2005; Kramvis & Kew, 2005).

HBV genotypes have distinct geographical distributions, which may alter over time and because of human migration patterns (Fung & Lok, 2004). Genotype A prevails in North America, Africa, India and North Western Europe (Norder *et al.*, 1993; Akuta & Kumada, 2005) and has a characteristic 6 nucleotide insert at the carboxy-terminal end of the core gene, which is absent in other genotypes (Kramvis *et al.*, 2005). Complete genomic analysis has led to the further classification of genotype A into two subgenotypes, A1 and A2. A1 differs from A2 in that it has distinct sequence characteristics in all ORF's and transcription regulatory elements (Kimbi *et al.*, 2004). Moreover, complete sequence analysis of HBV isolates belonging to subgenotype A1

have demonstrated unique sequence characteristics suggesting that A1 has been endemic to the South African Black population for a long time (Kimbi *et al*., 2004). Subgenotype A2 has been reported to be widely distributed in European countries and the USA, whereas A1 prevails in sub-Saharan Africa (Tanaka *et al*., 2004).

Two subgenotypes of genotype B exist, both with distinct geographical distributions. Subgenotype B1 predominates in Japan whilst B2, which has a genotype C recombinant region overlapping the precore/core ORF, is found in Asia (Sagauchi *et al*., 2002). Genotype C is also subgenotyped into two groups, C1 and C2 and is mainly found in Southeast Asia, Japan and Oceania, whilst genotype D is generally found worldwide but is more common in the Mediterranean countries (Kimbi *et al*., 2004; Akuta & Kumada, 2005).

Genotype E is restricted to Africa and genotype F is found in Central and South America amongst the aboriginal people (Kao, 2002; Sumi *et al*., 2002; Kimbi *et al*., 2004). Genotype G has been identified in France and North America (Kao, 2002), with genotype H predominating in Central America (Kimbi *et al*, 2004).

In regions where more than one genotype circulates, coinfection with different genotypes may occur in both children and adults as a result of multiple exposures or superinfection (Kramvis *et al*., 2005). This may be seen in genotype D which can coexist with genotype A in South Africa even though A is the predominant genotype.

There are currently four major HBV serotypes, or antigenic determinants of HBsAg based on antigenic heterogeneity (Kramvis *et al.*, 2005), namely *adw, ayw, adr*, and *ayr* (Kao, 2002, Kramvis *et al*., 2005). Generally, *adw* serotypes are found in genotypes A, B, C, F and G, whilst genomes encoding *adr* and *ayr* may also occur in genotype C (Kao, 2002). Serotype *ayw* is found in genotypes A-E (Kramvis *et al.,* 2005).

1.2 CHRONIC HBV INFECTION

Chronic hepatitis B infection can be defined as a necroinflammatory lesion of the liver that lasts longer than 6 months and is associated with the presence of HBV markers of replication and virus-induced liver disease (Brunetto *et al*., 1993). It is one of the leading causes of morbidity and mortality worldwide (Ozgenc *et al*., 2004). Despite the progress of mass vaccination programs in recent years to control HBV, there are still over 350 million chronically infected individuals worldwide (Papatheodoridis and Hadziyannis, 2001; Flink *et al*., 2005). The evolution of HBV infection into either acute or chronic infection is directed by both viral and host factors, which are involved in influencing host immunotolerance and susceptibility, as well as the likelihood of the virus persisting or being eliminated (Brunetto *et al*., 1995).

On the basis of virus-host interactions, the natural history of the infection can be divided into three phases (Chen, 1993):

- Immune tolerant phase: patients are HBeAg-positive and have high HBV DNA levels in the serum, are asymptomatic with normal serum alanine aminotransferase (ALT) levels and minimal histological activity in the liver.
- Immune clearance phase: a proportion of the asymptomatic carriers show symptoms of acute hepatitis B.
- Low-replicative or integration phase: seroconversion from HBeAg-positivity to anti-HBe positivity, with persistence of HBsAg and no liver disease.

Chronic HBV infection has a variable course and outcome. A large proportion of infected individuals have a milder course associated with a reduction in HBV replication, leading to a loss in serum HBV DNA and seroconversion of HBeAg to e antibody (anti-HBe) (Papatheodoridis and Hadziyannis, 2001; Sumi *et al*., 2002). When HBV replication ceases and is associated with HBeAg seroconversion to anti-HBe, there is a transition to asymptomatic carrier status (Brunetto *et al*., 1993).

Spontaneous reactivation is frequently seen during the course of chronic HBV infection and involves the reappearance of HBeAg and/or HBV DNA after a period in which they were negative, as well as a rise in serum ALT levels (Laskus *et al*., 1994). More severe forms are associated with a poorer outcome of infection. A subset of patients with HBeAg negative chronic hepatitis B, are found to have persistent viremia resulting from a continuation of viral replication. This form of HBV infection leads to chronic liver necroinflammation and progressive fibrosis, and is associated with the selection of replication competent pre-core mutants unable to produce HBeAg (Papatheodoridis and Hadziyannis, 2001).

It has also been suggested that viral persistence may be due to immune selection pressure of cytotoxic T cells and T helper cell responses in chronic HBV infection, which leads to mutations in HBeAg and human leukocyte antigen (HLA) class I and II restricted T cell epitopes (Cabrerizo *et al*., 1999).

1.2.1 Chronic HBV infection in children

The majority of chronic HBV carriers acquired their infections during childhood. Asia and Africa for example, where HBV is highly endemic, carry a high rate of infection in young children because of maternal-infant or early childhood transmission (Broderick and Jonas, 2003). In contrast, in Western developed countries, HBV infection is uncommon in children and is mostly a disease of adulthood (Sokal *et al*., 1998).

HBV infection acquired at an early age has three distinct phases; a high replicative immune tolerance phase and little liver inflammation, an immune response phase leading to inflammation and decreased viremia levels seen by a loss in HBeAg, and an immune surveillance phase with no or mild inflammation and low or undetectable replication (Lindh *et al*, 2001). In neonates, infection is usually asymptomatic. Infections are less likely to become chronic in neonates infected peri-natally, if the mother is anti-HBe positive (Broderick and Jonas, 2003). The proportion of individuals who do develop chronic HBV infection also varies with age of infection, as >90% of neonates, 1-5% of

adults and an intermediate frequency of young children will become chronically infected (Broderick and Jonas, 2003).

1.3 HBV-ASSOCIATED MEMBRANOUS NEPHROPATHY

Membranous nephropathy is a kidney disorder characterized by the thickening of the capillary walls of the glomerular basement membrane by immune complexes, leading to the disruption of kidney function and protein leak into the urine as a direct result of inflammation of the glomerulus (*Figure 1.7*).

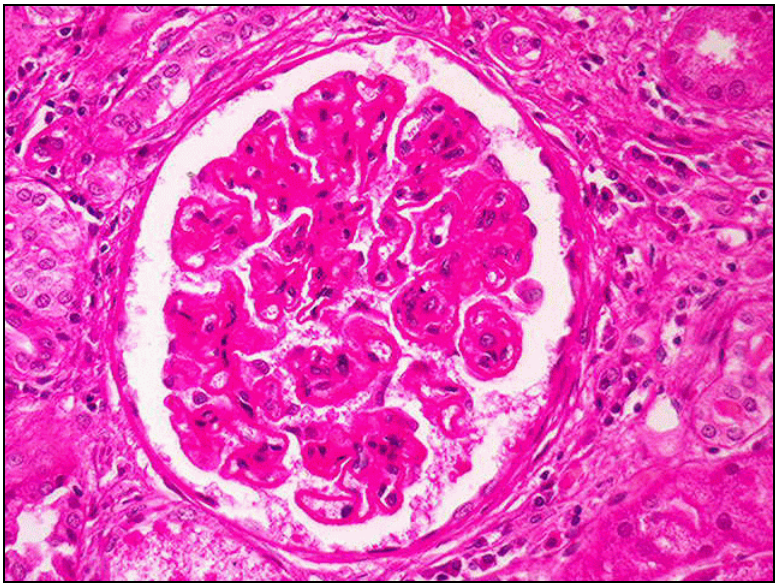


Figure 1.7: Haematoxylin and eosin stain of HBV-associated MN showing the thickened capillary walls of the glomerular basement membrane.

(http://oac.med.jhmi.edu/Pathology/Kidney/NephrSyn/082A_Full.html)

The cause of MN is unknown, but it is postulated to be associated with certain aetiological agents such as viruses, bacteria, parasites, autoimmune diseases and certain drugs (Dreyer, 1984).

Involvement of chronic HBV infection in the pathogenesis of human renal diseases has been reported since 1971 (Combes *et al*., 1971), and has been associated with clinical manifestations ranging from transient proteinuria to overt nephritis or nephrotic syndrome (Lai & Lai, 1991). This is possibly due to the continued release of viral antigens into the bloodstream during HBV infection, providing an ideal setting for the development of immune complex-mediated MN (Dreyer, 1984). Chronic HBV carriage has been strongly associated on clinical, epidemiological, and immunological evidence with the occurrence of MN, particularly in children (Combes *et al.*, 1971; Takekoshi *et al.*, 1978).

Studies have shown that HBV-associated nephropathy, particularly membranous nephropathy, is a major cause of nephrotic syndrome in Black children in South Africa (Wiggelinkhuizen *et al.*, 1983; Bhimma *et al.*, 1998; Bhimma *et al.*, 2002b). In a previous study they tested the hypothesis that family members and household contacts of children with HBV associated MN (HBVMN) would have a prevalence of HBV carriage higher than population controls and which would be associated with significant proteinuria. A high prevalence of HBV carrier-state (37%) and asymptomatic proteinuria (28%) in families and household contacts of children with HBVMN was shown (Bhimma *et al.*, 1999), but contrary to expectations there was no correlation among family

members and household contacts between HBV markers and abnormal proteinuria (Bhimma *et al.*, 1999).

1.4 INTERFERON TREATMENT FOR HBV INFECTION

1.4.1 Background

To control the spread of HBV infection in South Africa, the hepatitis B vaccine was incorporated into the Expanded Programme on Immunization (EPI) as part of routine childhood immunization, in 1995 (Mphahlele *et al*., 2002). Although these mass vaccination programs have begun to control the spread of HBV, therapeutic intervention is the only option for those with established chronic HBV-associated disease.

The use of interferon to inhibit viral growth was first demonstrated in 1976 by Greensberg (Greensberg *et al.*, 1976). Since then, its therapeutic value has been widely used in the treatment of chronic HBV infection, due to its ability to interfere with viral replication and production.

IFN, a naturally occurring cytokine in the body, has antiviral, antiproliferative, antifibrotic and immunomodulating activity (Manns, 2002; Liaw, 2003). The IFN pathway is initiated when a cell is infected by a virus. Viral gene products will induce the synthesis and secretion of IFNs, which bind to extracellular domains of cell surface receptors and activate IFN-stimulated genes (ISG), whose products will inhibit viral replication and activate a global antiviral state (Sen, 2001).

Interferons can be divided into two subfamilies, namely the type I family, of which IFNα, -β, -ω and -τ are structurally related members, and the type II family, of which IFNγ is a member (Sen, 2001). All type I IFNs are secreted proteins and IFNα in particular, formerly known as leukocyte IFN, has four conserved cysteine residues which form intramolecular disulphide bonds, necessary for maintaining the biological activity of the protein (Sen, 2001). Recombinant forms of IFNα, for example IFNα-2a and IFNα-2b, are used to treat chronic HBV infection.

IFNs immunomodulating effect includes mediating the expression of major histocompatability complex antigens, specifically that of the human leukocyte class I antigens on the surface of infected cells (Helvaci *et al*., 2004), and promoting the activity of macrophages and cytotoxic T cells to clear virally infected hepatocytes (Manns, 2002). Its antiviral activity involves the inhibition of viral penetration, replication and release from hepatocytes (Manns, 2002). Thus, the interaction of IFN with the cytokine cascade and T cell system is the key to its mode of action (Tilg, 1997).

It has been suggested that sustained response to HBV treatment can only be achieved by induction of the host immune response (Janssen, 2005). Therefore, IFN is theoretically an ideal treatment for HBV infection. This however, has not proved to be the case.

1.4.2 Response rates to IFN treatment

The short-term goal of any chronic HBV treatment is, first and foremost, to relieve any symptoms, reduce hepatic disease activity and to normalize ALT levels by making sure that there is a loss of HBeAg and/or HBV DNA (Liaw, 2003). The ultimate goal is to prevent cirrhosis of the liver and HCC (Manns, 2002).

Response to IFN has been defined as a loss in HBeAg and HBV DNA, but many responders continue to show HBsAg and HBV DNA in serum (Lindh *et al*, 2001). Response rates do however vary, with only 33% of patients treated with IFN losing HBeAg (Saltik-Temizel, 2004). Only 25-40% of chronic HBV infected patients treated with a 4 to 6 month course of IFNα therapy have been shown to achieve sustained remission (King *et al*., 2002). In 1993, a meta-analysis of all studies showed IFNα therapy was beneficial in some HBeAg-positive patients who were treated for three to six months (Al-Wakeel *et al.*, 1999). Treated patients were much more likely to seroconvert to anti-HBeAg and to show normalization of liver enzymes (ALT) than controls. Obviously, in the case of HBeAg-negative cases, seroconversion to anti-HBeAg would be irrelevant and treatment responses including decreased serum HBV DNA to levels undetectable by polymerase chain reaction assays and normalization of ALT values would be measured.

Children and adults have differences in immune tolerance and the rate of progression of liver disease because of differences in age at the time of infection, as well as differences

in the mode of transmission. Therefore studies of treatments for chronic HBV infection in adults cannot be directly extrapolated to children (Broderick & Jonas, 2003). The treatment of chronic HBV infection in children, with IFN is thus unclear. It is apparent that adult-onset HBV infection will respond better to treatment than neonatal infection (Manns, 2002).

IFNα treatment of chronic HBV infection in children in Western countries leads to a loss of HBV DNA or HBeAg seroconversion in 20 to 58% as compared with 8 to 17% in controls (Barbera *et al.*, 1994; Vajro *et al.*, 1996; Sokal *et al.*, 1998). This was demonstrated in a study carried out on 36 white children in whom a 50% response rate was seen after a 6 month course of therapy and was accompanied by a loss of HBV DNA and HBeAg and a decrease in serum ALT levels (Ruiz-Moreno *et al.*, 1991). Similar studies done on Chinese chronically infected children showed no differences in outcome between placebo and IFN treated patients (Lai *et al.*, 1987). Success rates are therefore not as high for the treatment of children in southeast Asian countries, most of whom were perinatally infected; only 3% to 17% clear HBV DNA or seroconvert from HBeAg to anti-HBe (Lai *et al.*, 1987).

Even though HBV is hyperendemic to sub-Saharan Africa, only a limited number of studies have been carried out to determine the efficacy of IFNα treatment of HBV infection in this area. In 1986, Dusheiko and his colleagues elucidated the efficacy of recombinant IFNα treatment on South African chronic HBV patients. All 28 patients tested were positive for HBsAg, HBeAg and HBV DNA at the onset of treatment.

However, at completion, inhibition of HBV replication and disappearance of HBeAg and HBsAg was only seen in 18% of patients (Dusheiko *et al*., 1986).

In a study, carried out by Bhimma and colleagues (Bhimma *et al.*, 2002a), twenty-four Black children with biopsy-proven HBV-associated nephropathy were recruited during the period April 1997 to June 1999 and treated with IFNα-2b for 16 weeks. Five children defaulted treatment and were therefore excluded from the study. A control group made up of 20 patients not receiving treatment was followed up for the same period of time. Response to treatment was defined as loss of HBeAg, decrease in proteinuria and prevention of deterioration in renal and liver function. By 40 weeks, ten (52.6%) of the treated children had responded to treatment with clearance of HBeAg. All of the responders demonstrated remission of proteinuria, 90% maintained normal renal function and 1 (10%) showed improvement of renal function. There was also a decrease of HBV DNA levels in this group. Nine patients did not clear HBeAg; none showed remission of proteinuria; 2 showed deterioration of renal function. The dominant HBV population in all 19 patients belonged to genotype A, the major genotype found in southern African Blacks. It was found that liver enzymes rose during treatment but subsequently declined irrespective of response. Only 5% of the control group showed spontaneous clearance of HBeAg, but none showed remission of proteinuria. It was therefore concluded that some Black children with HBV-associated nephropathy show accelerated clearance of HBeAg with remission of proteinuria following treatment with IFNα-2b.

1.4.3 Factors contributing to response

Considering the fact that IFN treatment is expensive, is administered by injection, may have side-effects and can be poorly tolerated, the ability to predict treatment response is important in a clinical setting (Kramvis & Kew, 2005). Viral heterogeneity as well as host factors such as age, sex, race and immune competence status may all affect response to IFN therapy (Brunetto *et al*., 1993).

1.4.3.1 Host Factors

Certain factors within the host may predict a high likelihood of a beneficial response to IFN therapy (Sokal *et al.*, 1998). These factors include high levels of ALT and low HBV DNA levels in the serum at start of treatment. According to Lai *et al* (1987), raised ALT levels in IFN treated patients reflect the hosts attempt to mount an immune response against the infection, whereas normal ALT levels reflect latent disease. In children, conflicting results have been found. In a study on Chinese children, low serum ALT levels and high HBV DNA levels seemed to predict low response to IFN therapy (Lai *et al.*, 1987), whereas other studies have demonstrated that children with high ALT levels correlated with a better response to IFN therapy (Sokal *et al.*, 1998; Moreno *et al.*, 1991). Other host factors that may play a role in predicting good response include female gender and active fibrosis on liver specimens in HBeAg-positive chronic HBV patients (King *et al*., 2002).

Together with conventional predictors of treatment response, single nucleotide polymorphisms (SNPs) may also be useful predictive markers for response to therapy. With regard to the host's genetic background, SNPs have been found to play a role in disease and treatment response via the host immune and antiviral response (King *et al.*, 2002). Specifically, polymorphisms in the IFN pathway have been identified which may play a role in IFN response in patients with chronic HBV infection.

1.4.3.2 Molecular Virological Factors

The molecular virological factors that contribute to the responsiveness of HBV infection to IFN treatment are largely unknown and may play a significant role in predicting whether IFN can be used effectively for treatment. To date the most important viral factor that has convincingly been shown to determine the response to IFN, is the pretreatment HBV DNA titre; the lower the titre the better the response (Aikawa *et al.*, 1995).

HBe antibody and HBV DNA-positive chronic HBV infection is a separate clinical entity from HBeAg-positive chronic HBV infection (Saruc *et al.*, 2003). Only 30-40% of HBeAg-positive patients and 10-50% of HBeAg-negative patients have been reported to respond to IFN therapy (Erhardt *et al.*, 2000). A number of studies have been carried out to address the significance of HBeAg status for the long term outcome of IFN therapy. Most studies have shown that in HBeAg-positive patients, low ALT levels, high HBV DNA replication and a long duration of disease is associated with low IFN response rates (Erhardt *et al.*, 2000). It has been reported by Lampertico *et al* (1997), that a sustained

virological and clinical response was achieved with prolonged IFNα treatment in HBeAg-negative or anti-HBe positive patients. Similarly, sustained response rates of 25-50% have been reported by other studies on HBeAg-negative patients (Aikawa *et al.,* 1995; Zhang *et al*., 1996). These results seem to correlate with a study in which approximately 30% of HBeAg-negative patients with chronic hepatitis B virus infection showed lasting suppression of infection after 24 months of IFN treatment (Lampertico *et al*., 2003). However, these data seem to be in disparity with a study in which HBeAg-negative status was said to be related to a high relapse rate and poor sustained response of 10% to IFN therapy (Aikawa *et al*., 1995). Another group concluded that a sustained response to IFN was independent of HBeAg status (Erhardt *et al.*, 2000).

HBeAg is coded for by the precore/core ORF and transcription of the precore/core mRNA is controlled by the basic core promoter (BCP). Therefore it has been postulated that mutations in either the BCP or the precore/core region could affect responsiveness of IFN treatment.

The presence of mutations within the BCP, namely, A1762T / G1764A and single substitutions at position 1751 through 1755, have been hypothesized to play a role in HBV replication and HBeAg synthesis (Zampino *et al*., 2002). Moreover, these mutations have been shown to correlate with response to IFN treatment in some studies (Erhardt *et al.*, 2000; Zampino *et al*., 2002) but not in others (Kao *et al.*, 2000; Hannoun *et al.*, 2002a). Low HBV DNA levels and elevated ALT levels in addition the BCP

mutations have been proposed to be favorable factors of response in IFN-induced anti-HBe seroconversion (Zampino *et al.*, 2002).

One study found that response to IFN had little to do with BCP mutations, but was merely as a result of viral loads, thus not supporting the hypothesis that BCP mutations are indicative of favorable IFN response rates (Shindo *et al.,* 1999).

The data on the relevance of precore mutants and their influence on the long term response to IFN are also inconclusive. In some studies precore mutants were considered to be necessary for response to IFN (Takeda *et al.*, 1990; Aikawa *et al.*, 1995; Lok *et al.*, 1995) . One such study reported a shift in pattern from wild-type to the precore mutant following IFN treatment in 6 out of 9 responding patients compared with 0 out of 9 non-responders, suggesting that long term IFN response is associated with the take-over of precore mutants (Lampertico *et al*., 1995). These data differ with respect to other studies which showed that precore mutants do not have prognostic value for virus elimination following IFN therapy in HBeAg-positive or HBeAg–negative patients (Xu *et al.*, 1992; Fattovich *et al.*, 1995). Numerous reports have also indicated that the precore mutant is resistant to IFN (Brunetto *et al*., 1993; Fattovich *et al*., 1995).

Because of selection pressure of IFN therapy, mutations may also arise in the polymerase gene of HBV. There are however no 'signature' mutations in this region which can be correlated with IFNα response (Chen *et al*., 2003). Likewise, because of the low emergence of mutations in the X region of HBV during IFN therapy, this region does not

seem to be a major determinant of response (Hannoun *et al*., 2002a). These differences in responsiveness to IFN treatment are probably the result of different genotypes of the virus and therefore an analysis of how various mutations influence the therapeutic response to IFN also requires knowledge about the genotype (Lindh, 1997).

In one study, a response rate of 41% compared with that of 15% was seen in genotype B compared with genotype C respectively, following IFNα-2b treatment (Kao *et al*., 2000). In a similar study, a higher response rate was observed in patients infected with genotype A than those infected with genotype D/E (Zhang *et al.,* 1996). Similarly, Erhardt *et al* (2000), reported that a study of 64 German patients revealed that the rate of IFN-induced HBeAg seroconversion was higher among patients infected with genotype A (37%) than those infected with genotype D (6%). Further investigation revealed that sustained response to IFN therapy was also higher in genotype A compared with that of genotype D infected patients (49% vs 26%) (Erhardt *et al*., 2005). In contrast, in a study carried out in Japan, IFN was given to 7 patients with prolonged HBV infection, and 4 of them responded. Of the 4 responders, one was infected with HBV genotype B and three with genotype C. HBsAg persisted in the remaining 3 patients all of whom were infected with HBV genotype A, and HBeAg remained positive in one of them (Kobayashi *et al.*, 2002). Following IFN treatment, genotype switching has been observed as a result of infection with a mixture of genotypes prior to treatment (Gerner *et al.*, 1998; Hannoun *et al.*, 2002a; Hannoun *et al.*, 2002b). The minor quasispecies populations were, however, not detected by either standard genotyping assays or direct sequencing but were detected using either a genotypic-specific polymerase chain reaction (PCR) and restriction

fragment length polymorphism (RFLP) or cloning (Kramvis & Kew, 2005). Genotype shifts suggest that genotypes are influenced by IFN differently (Hannoun *et al*., 2002b). IFN treatment may zone the immune system towards the major strain, thus allowing the minor strains to take over or increase (Hannoun *et al*., 2002b).

Thus, a thorough knowledge of the HBV strains circulating within a specific geographical region is required because studies from one geographical region cannot be directly extrapolated to regions where the circulating genotypes are different.

1.5 RATIONALE AND AIMS OF THE STUDY

Many studies have been undertaken to elucidate the role of HBV mutations and their subsequent effect on patient response to IFN therapy. The precore/core, polymerase and X genes in particular, have been sequenced and analyzed in patients treated with IFN. However, little is known about the effect, if any, of surface gene mutations on IFN response and *vice versa*.

The objective of this pilot study was therefore to sequence the complete S open reading frame of HBV isolated from South African Black children with membranous nephropathy before, during and after treatment with IFNα-2b, in order to determine whether:

- mutations in the S gene of HBV isolates are useful predictive markers for IFN response;
- the emergence of mutants during treatment influences the outcome;
- there are minor populations of mutant genomes that influence the outcome.

This will allow us to establish whether differences in the efficacy of IFNα therapy in this group of patients are due, at least partly, to viral heterogeneity in the S gene because host factors may also be important determinants.

Chapter 2: MATERIALS AND METHODS

2.1 PATIENTS

Twenty-four Black patients were recruited from the Renal Clinic at the King Edward VIII Hospital Durban, during the period April 1997 to June 1999. Nineteen of these completed the full course of therapy. The remaining 5 patients defaulted treatment, completing less than 80% of the course of treatment, and were excluded from the primary analysis. All patients recruited into the study had nephrotic syndrome diagnosed in accordance with the standard criteria (Barratt *et al.*, 1994); all were carriers of HBsAg and HBeAg for a minimum period of 6 months before entry into the study. Clinical examination and appropriate investigations were done to exclude other secondary causes of nephrotic syndrome (Bhimma *et al*., 2002a).

2.1.1 Interferon Treatment

This was a prospective, open-labeled, observational study of the use of IFNα-2b in Black African children. IFNα-2b (Intron A, Shering-Plough Co., USA) in a dose of 10 million units/m^2 (maximum 10 million units/dose) was administered subcutaneously 3 times per week by trained health care professionals to all study subjects. Before starting treatment, a test dose of 3 million units was given to all subjects in hospital with full resuscitation facilities available. Patients were treated for 16 weeks. Treatment dosage and adverse events were recorded by the health professional on a report card carried by the parent or guardian. Proteinuria, HBV status, liver function tests (including liver enzymes), and

renal function were recorded on weeks 8, 16 and 40 on the record cards. The card was monitored by the principal investigator and returned to him at the end of treatment. IFNα-2b therapy was discontinued if the child developed severe neurological symptoms (convulsions, severe psychiatric symptoms) or haematological disturbances (white cell count < 1.5 x 10^9/l; haemoglobin <7.0 x 10^9 g/l or platelets < 150 x 10^9/l) (Bhimma *et al.*, 2002a).

None of the patients had been treated with steroids or with cytotoxic agents. Symptomatic oedema was treated with salt restriction and diuretics. In those patients unresponsive to these measures, salt free albumin solution (20%) in combination with diuretics was used. Hypertension was controlled using angiotensin converting enzyme antagonists, thiazide diuretics, and calcium ion antagonists, singly or in combination.

2.1.2 Outcome Measures

These included HBV status, clinical criteria, and biochemical assessment of renal and liver function. Clinical criteria assessed were oedema, hypertension, and proteinuria. Histological assessment of renal and hepatic status on follow-up renal and liver biopsies was not undertaken because of failure to obtain ethical consent for these procedures from the University of Natal Research Ethics Committee (Bhimma *et al.*, 2002a).

2.1.3 HBV Determination

HBV status determination using 3rd generation Elisa and assays of HBV DNA using AMPLICOR to determine HBV genomes/ml were undertaken at study entry, 8, 16 and 40 weeks. Using AMPLICOR, the upper threshold was defined as >4 $x10^7$ genomes/ml and the lower limit as 400 genomes/ml. A virological response was identified as a reduction of HBV DNA to $<10^6$ copies/ml, including a 97% (1.5 logs) reduction of viremia (Lindh *et al.*, 2001).

All subjects also had hepatitis C virus (HCV) testing using both microparticle enzyme immunoassay (MEIA) for detection of antibodies (Abbott Imx ® HCC version 3.0) and reverse transcriptase polymerase chain reaction (RT-PCR) (Smuts & Kannemeyer, 1995). Human immunodeficiency virus (HIV) status was determined using Enzyme Linked Immunosorbent Assay (Abbott Laboratories recombinant HIV 1-2 ®, 3rd generation EIA) with confirmation by immunofluorescence assay (Viron ®) (Bhimma *et al.*, 2002a).

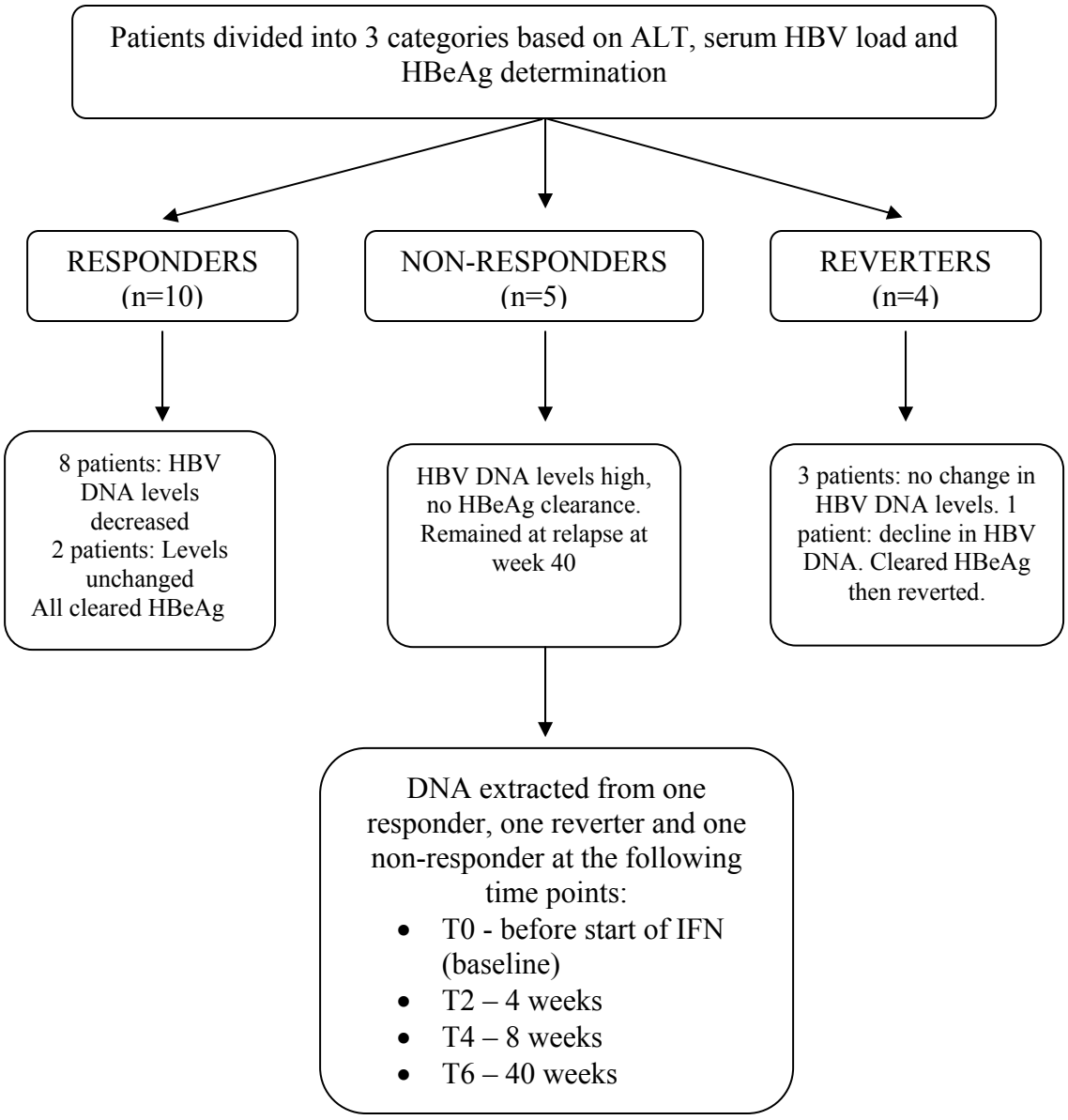


Figure 2.1: Flow diagram summarizing the serum samples used in the present study.

2.2 EXTRACTION OF HBV DNA FROM SERUM

Serum obtained from patients was used to extract HBV DNA using the QIAamp® DNA Blood Mini Kit (QIAGEN, GmbH, Hilden, Germany), as per manufacturer's instructions. This kit allows for the purification of total DNA, free of protein, nucleases and other contaminants or inhibitors, from blood, plasma, serum or other body fluids.

Before starting, the samples and the buffers were equilibrated to room temperature, and a heating block was pre-heated to 56ºC. 200 µl of the serum sample was added to a 1.5 ml Eppendorff tube, together with 20 µl QIAGEN Protease (Proteinase K). 200 µl Buffer AL was then added to the sample and the mixture pulse-vortexed for 15 sec to mix, followed by 10 min incubation at 56ºC. 200 µl of absolute ethanol was then added to the sample and again mixed by pulse-vortexing for 15 sec. The mixture was then carefully applied to a QIAamp Spin Column and centrifuged at 8 000 rpm for 1 min. The column was placed in a clean collection tube and 500 µl Buffer AW1 added. Centrifugation was again carried out at 8 000 rpm for 1 min and the column placed in a clean collection tube. After addition of 500 µl Buffer AW2, the QIAamp Spin Column was centrifuged at 14 000 rpm for 3 min. To eliminate any chance of Buffer AW2 carryover, an optional centrifugation step was carried out for an extra min at 14 000 rpm. The QIAamp Spin Column was then placed in a sterile 1.5ml Eppendorff tube, 50 µl best quality water (BQW) added directly to the column and incubated for 5 min at room temperature. The DNA was eluted by centrifugation for 1 min at 8 000 rpm and stored at -20ºC until needed.

In addition to the serum sample, a negative control consisting of BQW was included in the extraction procedure to check for contamination.

2.3 POLYMERASE CHAIN REACTION (PCR)

PCR is a highly specific and sensitive method designed to amplify large quantities of a DNA sequence without the use of cloning. The basic three steps of PCR involve a denaturation step in which double stranded DNA is denatured to single stranded DNA by heating to 94ºC, an annealing step in which the primers anneal to either side of the target sequence via their complementary bases and lastly, the extension of the target sequence by a DNA polymerase. Each of the aforementioned steps occurs at different temperatures in a thermal-cycler, resulting in an exponential increase in the target DNA.

2.3.1 Full-length PCR

The full genome of HBV was amplified from extracted DNA in a 25 µl reaction, on the Robocycler® Gradient 40 Temperature Cycler (Stratagene, Whitehead Scientific, La Jolla, CA) using the method described by Günther *et al* (1995).

Table 2.1: Oligonucleotide primers for full-length PCR

Primer	Nucleotide position	Primer Sequence*	Size of amplicon (bp)
P1.1 (+)	1821 - 1841	5' TTTTTCACCTCTGCCTAATCA 3'	3215
P2.1 (-)	1825 - 1806	5' AAAAAGTTGCATGGTGCAGG 3'	

Abbreviations: (+) sense, (-) anti-sense

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

Because the amplification was carried out as a 'hot-start' PCR, the reaction mixture consisted of two components:

- 5 µl of HBV DNA was amplified by PCR in a 15 µl reaction mix containing; 10.3 µl BQW, 2 µl 10X Expand High Fidelity Buffer with 1.5 mM $MgCl_2$, 0.2 µl 25 mM dNTP's and 1.25 µl each of the primers P1.1 (1821-1841 from *Eco*RI site) and P2.1 (1825-1806 from *Eco*RI site), respectively (*Table 2.1*) in a 0.5 ml Eppendorff tube. A drop of sterile mineral oil was added to each tube to prevent evaporation of the sample.
- The enzyme mix, made up in a separate tube, consisted of 3.75 µl BQW, 0.5 µl 10X Expand High Fidelity Buffer with 1.5 mM $MgCl_2$, and 0.75 µl Expand High Fidelity Enzyme mix (Roche Applied Science, Germany).

To perform a 'hot-start' PCR, the premix containing the DNA was denatured at 94ºC for 2 min and 40 sec, followed by the addition of 5 µl of the enzyme mix during the first annealing step of 58ºC for 1 min 30 sec. This was followed by 40 cycles of amplification

with a cycle profile of 94°C for 40 sec (denaturation), 58°C for 90 sec (annealing), and 68°C for 3 min with an increment of 2 min after every 10 cycles (extension).

A positive control as well as a negative control consisting of a HBV-positive sample and BQW, respectively was included in all PCR reactions.

2.3.2 Amplification of the Surface Region of HBV

A nested PCR was carried out to amplify the entire S region of HBV (preS1/S2/S region). This PCR was carried out on the GeneAmp PCR System 9600 Perkin Elmer (Biotechnology, U.S.A).

First round PCR consisted of 22.5 µl of a reaction mixture containing 15.4 µl BQW, 2.5 µl 10X Promega Buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl 40mM dNTP's (10 mM each dNTP), 1.25 µl each of the outer primers, 2408F and 1327R, respectively (*Table 2.2*), and 0.1 µl Promega Taq DNA Polymerase (Promega, Madison, WI) in a 0.2 ml Eppendorff tube. To this mixture, 2.5 µl of the HBV DNA was added to bring the total reaction mixture to 25 µl.

PCR cycling for the first round involved 40 cycles of denaturation at 94ºC for 60 sec, annealing at 66ºC for 5 mins and extension at 72ºC for 3min. 5 µl of the above reaction was then added to a second round mixture containing; 30.8 µl BQW, 5 µl 10X Promega Buffer, 3 µl 25 mM $MgCl_2$, 1 µl 40 mM dNTP's (10 mM each dNTP), 2.5 µl each of the

inner primers 2800F and 1011R, respectively (*Table 2.2*), and 0.2 µl Promega Taq DNA Polymerase (Promega, Madison, WI) in a 0.2 ml Eppendorff tube. The second round cycling profile consisted of 40 cycles of denaturation at 94ºC for 60 sec, annealing at 66ºC for 1 min and extension at 72ºC for 1 and a half mins.

Table 2.2: Oligonucleotide primers for surface gene PCR

Primer	Nucleotide Position	Primer Sequence*	Size of amplicon (bp)
Outer Primers			
2408(+)	2408 - 2438	5' TCTCAATCGCCGCGTCG CAGAAGATCTCAA 3'	2119
1327(-)	1327 - 1301	5' CGATGAGTTTTGCTCCA GACCGGCTGC 3'	
Inner Primers			
2800(+)	2800 - 2834	5' CACGTAGCGCATCATTTT GCGGGTCACCATATTCT 3'	1411
1011(-)	1011 - 977	5' CAAAAGACCACAATTCTT TGACATACTTTCCAAT 3'	

Abbreviations: (+) sense, (-) anti-sense

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

2.4 VISUALIZATION OF PCR PRODUCTS

Following PCR, the resulting DNA products were separated by size via electrophoresis on a 1% agarose gel containing ethidium bromide (*Appendix D1*). Sizes of PCR products were estimated relative to the migration patterns of a 1 kb DNA ladder (Promega, Madison, WI, USA) for the full-length PCR products and a 100 bp DNA ladder (Promega, Madison, WI, USA) for the surface gene PCR products. Products were visualized by placing the gel under ultraviolet light.

2.5 CLONING OF HBV DNA

The TOPO® XL PCR Cloning Kit (Invitrogen, Carlsbad, CA), is a quick (5 min), efficient, one-step cloning method for cloning PCR products. The TOPO® Cloning Reaction can be transferred directly into competent cells by chemical means. Recombinants are then selected by the disruption of the lethal *E.coli* gene, *ccd*B. Therefore, if the PCR product has correctly ligated to the vector containing the *ccd*B gene, expression of the gene is disrupted, allowing growth of positive recombinants upon plating.

2.5.1 Preparation of PCR products for cloning

2.5.1.1 Addition of 3'A Overhangs

Prior to cloning, 3' deoxyadenosine (A) overhangs needed to be incorporated onto the full-length PCR product. This was achieved by adding 1 µl TaKaRa Ex Taq DNA Polymerase (TaKaRa Bio Inc., Japan) to the product and the incubating for 10 min at 72ºC under mineral oil. As this enzyme has nontemplate-dependent terminal transferase activity, it is able to add single A's to the 3' end of the PCR product generated after full-length PCR. The plasmid vector (pCR®-XL-TOPO®) that was supplied in the TOPO® XL PCR Cloning Kit, is then linearized with the single 5' deoxythymidine (T) overhangs. Thus the addition of the A' overhangs onto the PCR product allows it to insert and ligate with the vector. This step was only needed for the full-length PCR products amplified using Expand High Fidelity Enzyme (Roche Applied Science, Germany) because this enzyme does not add on A' overhangs.

2.5.1.2 Purification of PCR products

The amplified DNA was purified using the MinElute® Reaction Clean-up Kit (QIAGEN, GmbH, Hilden, Germany), in order to remove any enzymes, salts or nucleotides, which would affect the cloning efficiency. Following manufacturer's instructions, the entire volume of PCR product was transferred to a new sterile Eppendorff tube. 300 µl Buffer ERC was then added and the sample and votexed to mix. A MinElute column which adsorbs all the DNA to the silica-membrane, was then placed in a collection tube, all the

sample was applied to the column and the mixture was centrifuged for 1 min at 14 000 rpm. The flow-through was discarded and the column placed back in the collection tube. 750 µl Buffer PE was then added and the sample was again centrifuged under the same conditions as before to ensure all impurities were washed away. To dry the column, the centrifugation step was once again repeated for 1 min at 14 000 rpm. The column was the placed in a new sterile 1.5ml Eppendorff tube and the collection tube discarded. 10 µl BQW was added to the column and incubated for 1 min to allow the water to absorb. This was followed by a centrifugation step of 1 min at 14 000 rpm to elute the DNA from the column, proceeding directly to the purification of the product from a gel.

2.5.2 Gel Purification of PCR Product

Purified amplicons were electrophoresed on a 0.8% crystal violet agarose gel (*Appendix D1*) together with a 1 kb and 100 bp molecular weight marker (Promega, Madison, WI, USA). 40 µl of the PCR product was loaded onto the gel with 8 µl 6X Crystal Violet Loading Buffer and allowed to run at ~53 volts for 1½ to 2 hours, or once the crystal violet in the gel had run about a quarter of the way up the gel.

To excise the PCR product, the buffer was poured off and the gel placed on a fluorescent light box to help visualize the fragments. Using a new surgical blade, the band was carefully excised from the gel and placed into a sterile 1.5 ml Eppendorff tube. The volume of the agarose pieces was estimated by weighing the gel (1mg ~ 1µl), and 2.5 times this volume of 6.6 M sodium iodide was added. By periodically shaking the tube

vigorously and incubating at 45ºC on a hot block, the agarose was completely dissolved in approximately 2 min. 1.5 volumes of Binding Buffer was then added to the tube at room temperature and mixed well. To isolate the PCR product from the agarose, all the above mixture was loaded onto an assembled S.N.A.P.™ purification column (A) and collection vial (B). This was then centrifuged at 4 000 rpm in a microcentrifuge for 30 sec at room temperature. The liquid in the collection vial was then poured back into the column and again centrifuged for 30 sec at 4 000 rpm. The former step was repeated a third time to ensure all the DNA had bound to the column. After the third centrifugation, the liquid in the collection tube was discarded. 400 µl of 1X Final Wash was then added to the column and centrifuged as before for 30 sec. This step was repeated (400 µl new Final Wash) and the liquid in the collection tube (800 µl) was discarded. To dry the column resin, the column was centrifuged again at 14 000 rpm for 2 min, thereafter the column was transferred to a sterile 1.5 ml Eppendorff tube. To elute the DNA from the column, 40 µl TE buffer was added directly to the column and incubated for 1 min at room temperature to allow the buffer to absorb into the column. This was followed by a final centrifugation at 14 000 rpm for 1 min to elute the DNA from the column, after which the tube was placed on ice before proceeding to the next step.

2.5.3 TOPO® Cloning and Transformation

To clone the PCR product into the pCR®-XL-TOPO® vector, a 5 µl TOPO® Cloning reaction was set up. 4 µl gel-purified PCR product was added to 1 µl pCR®-XL-TOPO® vector in a sterile Eppendorff tube. The reaction was mixed by gently tapping with a finger and then incubated for 5 min at room temperature. After the 5 min incubation, 1 µl of the 6X TOPO® Cloning Stop Solution was added and mixed by gently tapping for several sec at room temperature. The tube was then briefly centrifuged and placed on ice.

To transform One Shot® TOP10 chemically competent cells, 2 µl of the TOPO® Cloning reaction was added into a vial of One Shot® TOP10 cells and gently mixed. This reaction was then incubated for 30 min on ice. After the incubation, the cells were heat-shocked for 30 sec at 42ºC without shaking, and then immediately transferred to ice for 2 min. 250 µl of room temperature S.O.C. medium was then added followed by a 1 hour incubation at 37ºC on a shaker. Following incubation, the tube was placed on ice.

50-150 µl from each transformation was then spread, using a glass spreader and aseptic techniques, on a prewarmed Luria-Bertani (LB) agar plate containing 50 µg/ml of the antibiotic kanamycin (Roche Diagnostics, GmbH, Mannheim, Germany) (*Appendix D1*). The plates were incubated overnight at 37ºC to allow for bacterial growth.

Individual cloned colonies, approximately 40 at a time, were aseptically restreaked using a sterile loop, onto new LB-Kanamycin plates and allowed to grow for 24 hours. Plates were then stored at 4ºC.

2.6 ISOLATION OF CLONED DNA

HBV DNA was extracted from the plasmid using the GenElute™ Plasmid MiniPrep Kit (Sigma-Aldrich, St Louis, MO, USA). This method allows for the isolation of plasmid DNA from recombinant *E.coli* cultures. Prior to extraction, bacteria from the LB-Kanamycin plates were inoculated into a 15 ml nunc tube containing 5 ml LB media and 5 µl Kanamycin and shaken overnight at 37ºC. After 24 hours, the overnight culture was spun for 15 min in a centrifuge at 5 000 rpm to pellet the cells. The cells were then resuspended in 200 µl Resuspension Solution by vigorously pipetting up and down, and transferred to a sterile 1.5 ml Eppendorff tube. 200 µl Lysis Solution was added to lyse the resuspended cells and release the DNA, inverted gently to mix and allowed to clear for 5 min. 350 µl Neutralization Solution was then added and inverted 4 to 6 times to mix, in order to precipitate cell debris. The debris was pelleted out by centrifuging the mixture for 10 min at 14 000 rpm.

To prepare the binding column in order to allow maximum binding of DNA, 500 µl Column Preparation Solution was added to the binding column in a collection tube, spun for 1 min at 14 000 rpm and the flow-through discarded. The plasmid DNA was then bound to the column by transferring all the cleared lysate into the binding column and

spinning for 1 min at 14 000 rpm. The flow-through was discarded and 500 µl Optional Wash Solution added to the column to remove all contaminants. The column was spun under the same conditions as before and the flow-through discarded. 750 µl Wash Solution was added, spun as before to discard flow-through and then spun an extra min at 14 000 rpm to dry the column.

To elute the purified plasmid DNA, the column was transferred to a new 1.5 ml collection tube and 50 µl BQW was added directly to the column. This was followed by a final spin at 14 000 rpm for 1 min. The concentrated plasmid DNA could be stored at -20ºC until needed. A negative control consisting of only LB-Kanamycin was included in the procedure to ensure the sterility of the media.

2.7 DIGESTION OF ISOLATED PLASMID DNA

2.7.1 Restriction Endonucleases

Restriction enzymes or restriction endonucleases, cleave DNA at a very specific site or recognition sequence. The recognition sequence is often a 6 base pair palindromic sequence, but some restriction enzymes recognize 4 or even 8 base pair sequences. Because on this sequence specificity, restriction enzymes are used to further characterize a particular DNA molecule by cutting the DNA into discrete fragments. The restricted DNA fragments can then be resolved by gel electrophoresis and the pattern the DNA fragments generate, produce a DNA 'fingerprint'.

2.7.1.1 *Eco*RI Digestion

Plasmid DNA was treated with the restriction enzyme *Eco*RI (Promega, Madison, WI), to check for the integration of the PCR product into the plasmid. A master mix of 4.9 µl BQW, 0.1 µl BSA, 1 µl Promega *Eco*RI Buffer and 1 µl Promega *Eco*RI enzyme (Promega, Madison, WI) per sample was added to 3 µl of the plasmid DNA. 1 drop of oil was added to the tube to prevent evaporation of sample. Digestion was carried out on a 37ºC hot block for 2 hours, after which 4 µl bromophenol blue (Merck, Darmstadt, Germany) loading dye was added to stop the reaction. The result of the digestion was visualised by running 10 µl of the sample overnight at ~30V on a 1% agarose gel, together with 1 kb and 100 bp molecular weight markers (Promega, Madison, WI, USA).

2.8 GENOTYPING

Lindh genotyping is a novel method for genotyping HBV, making use of restriction fragment length polymorphism (RFLP), using the restriction enzymes *Tsp509*I (recognizes AATT) and *Hinf*I (recognizes GANTC).

2.8.1 PCR

To amplify the DNA prior to genotyping, a 100 µl PCR reaction was set up in a 0.2 µl Eppendorff tube containing the following components: 64.8 µl BQW, 10 µl 10X NH_4 reaction buffer, 6 µl 50 mM $MgCl_2$, 8 µl 20 mM dNTP's, 5 µl each of the primers P7 and

P8 (*Table 2.3*), 0.2 µl 5u/µl Biotaq™ DNA Polymerase (Bioline, GmbH, Germany). To this reaction mix, 1 µl DNA was added.

Table 2.3: Oligonucleotide primers for P7/P8 genotyping PCR

Primer	Nucleotide position	Primer Sequence*	Size of Amplicons (bp)
P7(+)	256 - 278	5' GTGGTGGACTTCTCTCAATTTTC 3'	541
P8(-)	796 -776	5' CGGTATAAAGGGACTCACGAT 3'	

Abbreviations: (+) sense, (-) anti-sense

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

Amplification was carried out on the Eppendorf Mastercycler® Gradient (Eppendorf, Hamburg, Germany). An initial denaturation step of 94ºC for 3 min was followed by amplification of the above reaction for 40 cycles with the following parameters: denaturation at 94ºC for 45 sec, annealing at 53ºC for 1 min and extension at 72ºC for 1 and a half mins.

2.8.2 RFLP assay for Genotyping

A master mix was prepared for each of the enzymes, *Hinf* I (Promega, Madison, WI) and *Tsp* 509I (New England Biolabs, Beverly, MA), used for genotyping (*Table 2.4*).

Table 2.4: Master mixes for genotyping HBV clones

	***Hinf* I (in µl)**	***Tsp* 509I (in µl)**
BQW	2.3	2.5
10X Buffer	2	2
Bovine serum albumin	0.2	/
Restriction enzyme	0.5	0.5

5 µl of the each of the master mixes was added to two separate 0.5 ml Eppendorff tubes, followed by 15 µl of the PCR product. A drop of sterile mineral oil was added to each tube to prevent evaporation of the product. Both reactions were incubated for 3 hours; *Hinf* I at 37ºC and *Tsp* 509I at 65ºC. After incubation, the reaction was terminated by the addition of 4 µl loading dye. 10 µl of the sample was then run on a 3% ethidium bromide-stained composite agarose gel (*Appendix D1*) overnight at ~30V, alongside 100 bp and 1 kb molecular weight marker (Promega, Madison, WI, USA). The resulting restricted fragments were visualized under UV light.

2.9 SUBGENOTYPING OF HBV

In order to distinguish between subgenotypes A1 and A2 the method described by Kew *et al* (2005) was used. The following PCR reaction mix was set up in a 0.2 µl Eppendorff tube: 33.3 µl BQW, 5 µl 10X NH_4 reaction buffer, 1.5 µl 50 mM $MgCl_2$, 4 µl 10 mM dNTP's, 0.5 µl each of the primers 521F and 1192R (*Table 2.5*)and 0.2 µl 5u/µl Biotaq™

DNA Polymerase (Bioline, GmbH, Germany). To this reaction mix, 5 µl DNA was added.

Table 2.5: Oligonucleotide primers for subgenotyping of HBV

Primer	Nucleotide Position	Primer Sequence*	Size of amplicons (bp)
521(+)	521 - 541	5'CCTGCACGACTCCTGCTCAA 3'	671
1192(-)	1192 -1173	5'CGTCAGCAAACACTTGGCAC 3'	

Abbreviations: (+) sense, (-) anti-sense

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

An initial denaturation step of 94ºC for 3 min was followed by 40 cycles of denaturation at 94ºC for 45 sec, annealing at 60ºC for 1 min and extension at 72ºC for 1 and a half min on the Eppendorf Mastercycler® Gradient (Eppendorf, Hamburg, Germany).

To subgenotype HBV, the following master mix was prepared: 7.5 µl BQW, 2 µl 10X Buffer and 0.5 µl of the restriction enzyme *Stu*I (Promega, Madison, WI). 10 µl of this reaction mix was dispensed into a 0.5 ml Eppendorff containing 10 µl of the PCR product. 1 drop of sterile mineral oil was added to the mixture, and then incubated at 37ºC for 1 hour on a hot block. The reaction was terminated by the addition of 4 µl loading dye. To visualize the restricted bands, 10 µl of the sample was then run on a 3% composite agarose gel overnight at ~30V alongside a 100 bp and 1 kb molecular weight marker (Promega, Madison, WI, USA).

2.10 SEQUENCING OF HBV DNA

The clones containing the correct size amplicons were prepared for sequencing using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA). Sequencing was carried out on a 377 DNA automated sequencer (Applied Biosystems, Inc) using HBV-specific primers (*Table 2.6*). All sequences were analysed in both the forward and reverse directions.

Table 2.6: Oligonucleotide primers and annealing temperatures for sequencing the surface gene of HBV

Primer	Sequence	Annealing Temperature
116(+)	5’ TATCGTCAATCTTCTCG 3’	50°C
521(+)	5’ CCTGCACGACTCCTGCTCAA 3’	60°C
840(+)	5’ CTCCAATTTGTCCTGGTTATC 3’	50°C
2810(+)	5’ CATCATTTTGTGGGTCACCAT 3’	66°C
T7(+)	5’ GTAATACGACTACTATAGGGC 3’	50°C
270(-)	5’ AGAGAAGTCCACCACGAGTCTAGA 3’	55°C
455(-)	5’ CAAGGTATGTTGCCCGTTTG 3’	55°C
1011(-)	5’ CAAAAGACCACAATTCTTTGACATACTTTCCAAT 3’	66°C
898(-)	5’ GTCGTGCAGGTTTTGCAG 3’	50°C
M13(-)	5’ AGCGGATAACAATTTCACACAGG 3’	50°C

Abbreviations: (+) sense, (-) anti-sense

2.11 PHYLOGENETIC ANALYSES

Complete surface gene sequences from the responder, reverter and non-responder were compared with corresponding sequences of HBV from GenBank. Multiple sequence alignments were carried out using Dambe (Xia, 2000). The alignments were edited manually in GeneDoc (Nicholas, 1997) and fed into PHYLIP (Phylogeny inference package) version 3.5c (Felsenstein, 1995). DNAML (maximum likelihood) alone and DNADIST consecutively with NEIGHBOR (neighbour-joining) were used to generate dendograms. SEQBOOT, DNADIST and NEIGHBOR were used for bootstrapping of 1000 data sets. CONSENSE was used to compute a consensus tree. Finally, trees were visualized using the TreeView Win 32 software program (Page, 1996).

2.12 STATISTICAL ANALYSES

The relevant statistical test, namely one-way ANOVA's, were used to analyze the nucleotide and amino acid divergences between the responder, reverter and non-responder at the different times of sampling.

2.13 ETHICAL CONSIDERATIONS

The protocol was approved by the Biomedical Research Ethics Committee, Faculty of Medicine, Nelson R. Mandela School of Medicine, University of Natal, Reference

number: E114/04 (*Appendix A2*) and informed consent was obtained from the parent or guardian for entry into the study. Approval was also obtained by the Human Research Ethics Committee, protocol number M050705 (*Appendix A1*), University of the Witwatersrand, for the use of serum samples in the present study.

Chapter 3: RESULTS

HBV DNA from a responder, reverter and non-responder were extracted from the serum samples obtained from Black children with membranous nephropathy treated with IFN for 16 weeks. At 40 weeks of follow up, the responder had cleared HBeAg whereas the reverter had cleared HBeAg during treatment but then reverted to being HBeAg-positive. The non-responder failed to clear HBeAg. Clearance of HBeAg was associated with the appearance of anti-HBe. None of the patients cleared HBsAg. All children had high HBV DNA levels on commencement of treatment (>2 x 10^{6} genome/ml) (Bhimma *et al*., 2002a). The responder showed a decline in HBV DNA levels at 40 weeks and the levels were unchanged in the non-responder. The reverter showed a decline in HBV DNA levels at 16 weeks but reverted to pretreatment levels after the cessation of treatment (Bhimma *et al*., 2002a).

Full genome amplification of the non-responder (NON-RESP) and surface gene amplification of the responder (RESP) and reverter (REV) was carried out at time points before (T0), during (T2 and T4, 4 and 16 weeks after the initiation of treatment, respectively) and after treatment (T6, 40 weeks after the initiation of treatment).

3.1 DETECTION OF AMPLIFIED PCR PRODUCTS

3.1.1 Complete Genome Amplification

The method for complete genome amplification of Günther *et al* (1995) could not be used to amplify HBV DNA from the serum of IFN-treated patients and therefore the method was modified and adapted. Because the concentration of DNA was found to be too high in our samples, the extracted DNA was diluted 1:2 or 1:5 with BQW. Moreover, instead of using an annealing temperature of 57ºC, a gradient ranging from 57º to 62ºC was applied. Using these modifications, the complete genomes of HBV isolates from the non-responder were successfully amplified at the various time points, T0, T2, T4 and T6. The correct size amplicons were visualized on a 1% agarose gel as a 3.2 kb band (*Figure 3.1*). The optimum amplification was obtained using a dilution of the DNA extract of 1:2 and an annealing temperature of either 57º or 60º C.

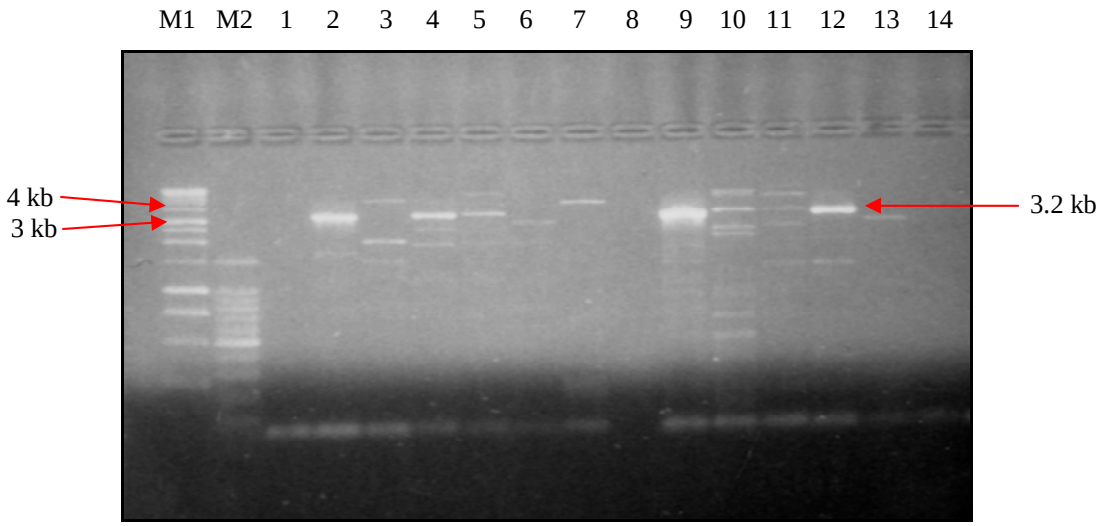


Figure 3.1: Identification of HBV full-length genomes from a non-responder as seen by a 3.2 kb band on a 1% ethidium bromide-stained agarose gel. M1 = 1 kb molecular weight marker; M2 = 100 bp molecular weight marker; Lanes 1, 8, 14 = BQW blank; lanes 2-7 = HBV DNA at a 1:5 dilution corresponding to annealing temperatures ranging from 57ºC – 62ºC; lanes 9 – 13 = HBV DNA at a 1:2 dilution corresponding to annealing temperatures ranging from 57ºC – 61ºC.

Where HBV DNA concentrations were not high enough and full genome amplification was not possible, as was the case for the responder and reverter, only the preS1/S2/S-gene was amplified at T0, T2, T4 and T6 for these samples.

3.1.2 Surface Gene Amplification

In order to optimize the surface gene PCR, amplification was attempted on two different machines, the GeneAmp PCR System 9600 Perkin Elmer (Biotechnology, U.S.A) and the

Eppendorf Mastercycler® Gradient (Eppendorf, Hamburg, Germany). After amplifying the HBV DNA extracted from the serum samples obtained from the responder, at all time points, on both machines, it was found that the Perkin Elmer gave better amplification and was therefore used for all subsequent experiments. Bands of the appropriate size, 2119 bp for first round PCR and 1411 bp for second round PCR from the responder and the reverter were visualized after electrophoresis on a 1% agarose gel (*Figure 3.2*).

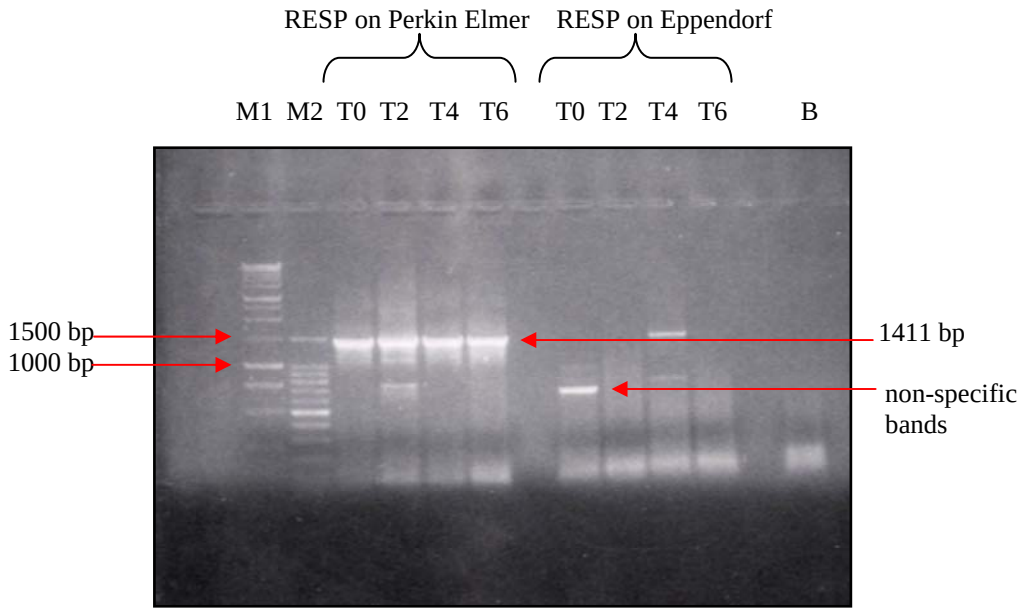


Figure 3.2: Comparison of second round S-region PCR from a responder after amplification on the GeneAmp PCR System 9600 Perkin Elmer (Biotechnology, U.S.A) and Eppendorf Mastercycler® Gradient (Eppendorf, Hamburg, Germany). M1 = 1 kb molecular weight marker; M2 = 100 bp molecular weight marker. Amplification of the S region on the Perkin Elmer resulted in the correct size amplicons, namely 1411 bp for all time points (T0, T2, T4, T6) whilst amplification on the Eppendorf resulted in non-specific binding of the primers. B = BQW blank.

All full genome amplicons from the non-responder and surface genes amplicons from the responder and reverter, at all time points, were cloned into a pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA).

3.2 IDENTIFICATION OF POSITIVE CLONES

3.2.1 *Eco*RI Digestion

Following TOPO® XL cloning and the extraction of plasmids, samples were digested with the restriction enzyme *Eco*RI (Promega, Madison, WI) to check whether the PCR amplicons were inserted correctly into the plasmids. After overnight electrophoresis on an ethidium bromide-stained 1% agarose gel, the correct sized amplicons were identified: for full-length clones, by a 3.5 kb plasmid band and a 1.8 kb and 1.4 kb band (*Figure 3.3*) and for the surface region, positive clones were visualized by a restriction pattern of 3.5 kb, 1 kb and 400 bp bands (*Figure 3.4)*.

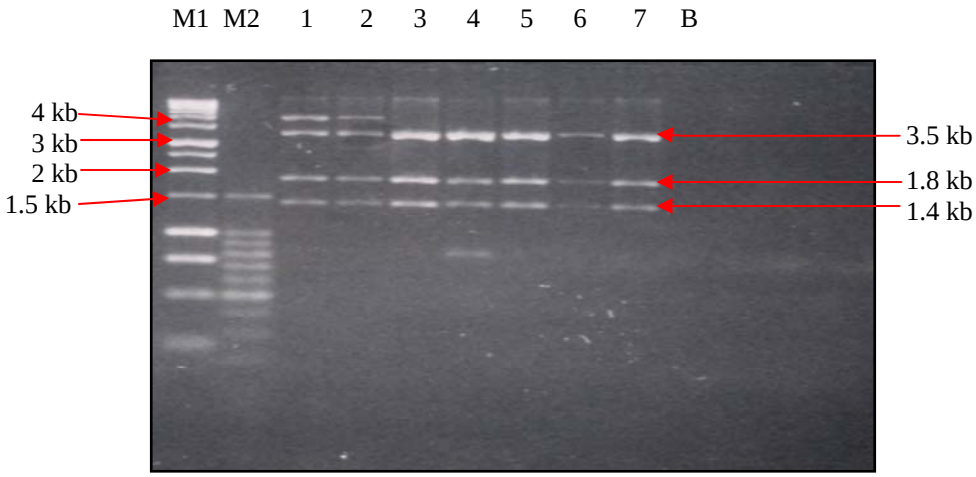


Figure 3.3: Visualisation of full-length HBV positive clones from a non-responder at T0, after *Eco*RI (Promega, Madison, WI) digestion for 2 hours. M1 = 1 kb molecular weight marker; M2 = 100 bp molecular weight marker; lanes 1-7 = NON-RESP positive clones; B = BQW blank.

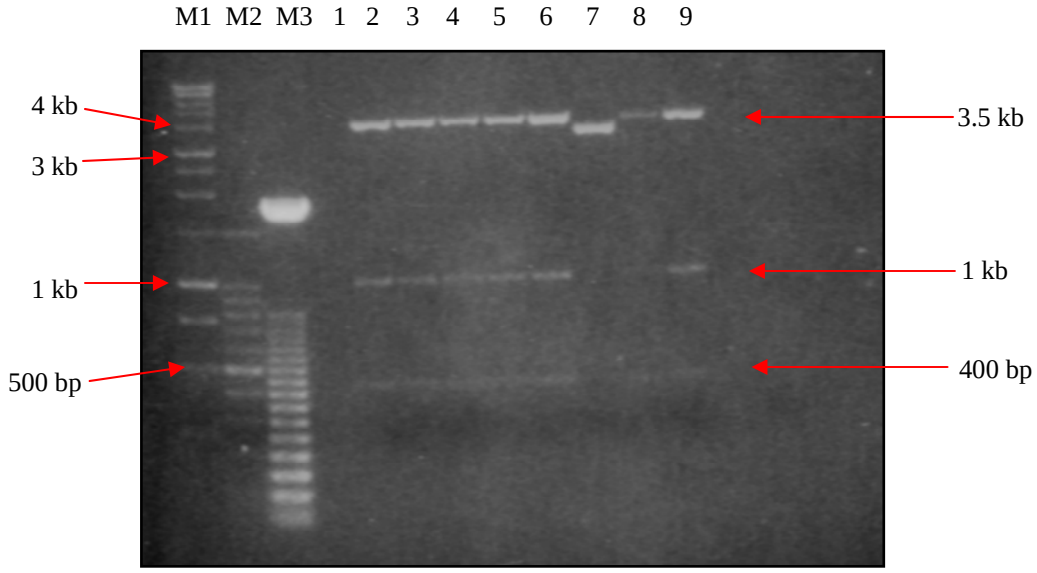


Figure 3.4: Visualisation of HBV surface gene positive clones from a responder at T4. M1 = 1 kb molecular weight marker; M2 = 100 bp molecular weight marker; M3 = 50 bp molecular weight marker; lane 1= BQW blank; lanes 2-6, 8-9 = positive clones for HBV surface gene; lanes 7 = negative clone not containing surface gene.

Following the identification of positive clones, the full-length and surface gene clones were genotyped and subgenotyped using RFLP analysis.

3.3 GENOTYPING

3.3.1 RFLP Analysis of Clones

Genotyping of HBV clones relies on the amplification of a region of the S gene of HBV, followed by the digestion of amplicons by restriction enzymes and RFLP analysis. Nucleotides 256 to 796 of the surface gene of HBV were successfully amplified from the full-length and the surface gene clones as shown by the presence of a 541bp band on a 1% agarose gel (*Figure 3.5*).

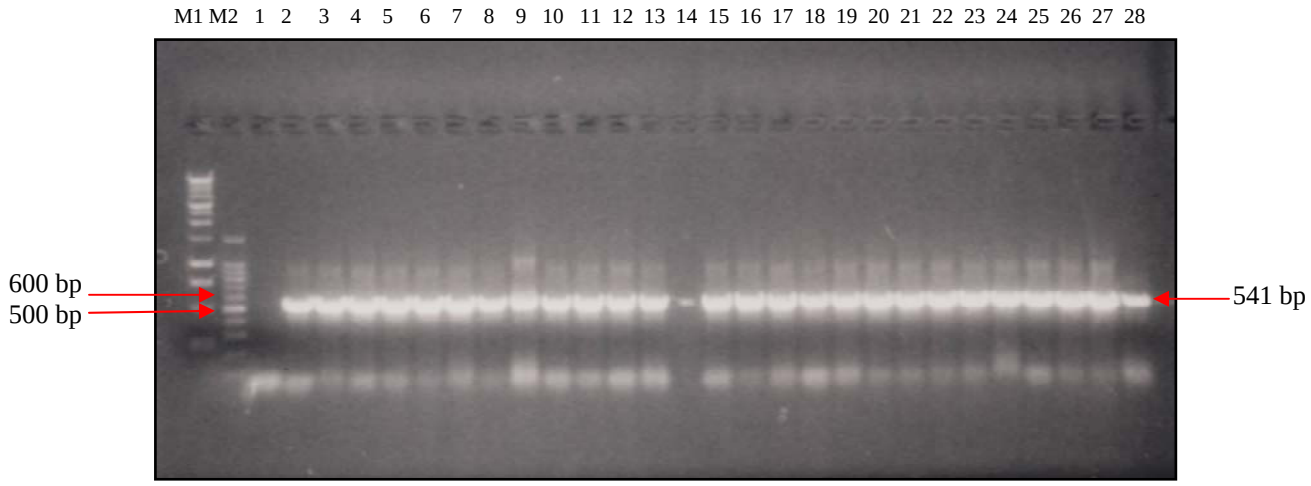


Figure 3.5: Identification of HBV surface gene PCR amplicons for genotyping of a reverter as seen by a 541 bp band on a 1% ethidium bromide-stained agarose gel. M1 = 1 kb molecular weight marker; M2 = 100 bp molecular weight marker; Lane 1 = BQW blank; lanes 2-13 and 15-28 = positive PCR amplicons; lane 14 = negative for PCR product.

The PCR products were then digested using restriction enzymes *Hinf* I and *Tsp* 509I. The RFLP pattern for all clones corresponded to that for genotype A: 274 bp, 252 bp and 15 bp fragments for *Hinf* I and 207 bp, 126 bp, 109 bp, 47 bp and 16 bp fragments for *Tsp 509*I (Lindh *et al.*, 1997) (*Figure 3.6*). Fragments smaller than 40 bp could not be visualized on the agarose gel, because of their small size.

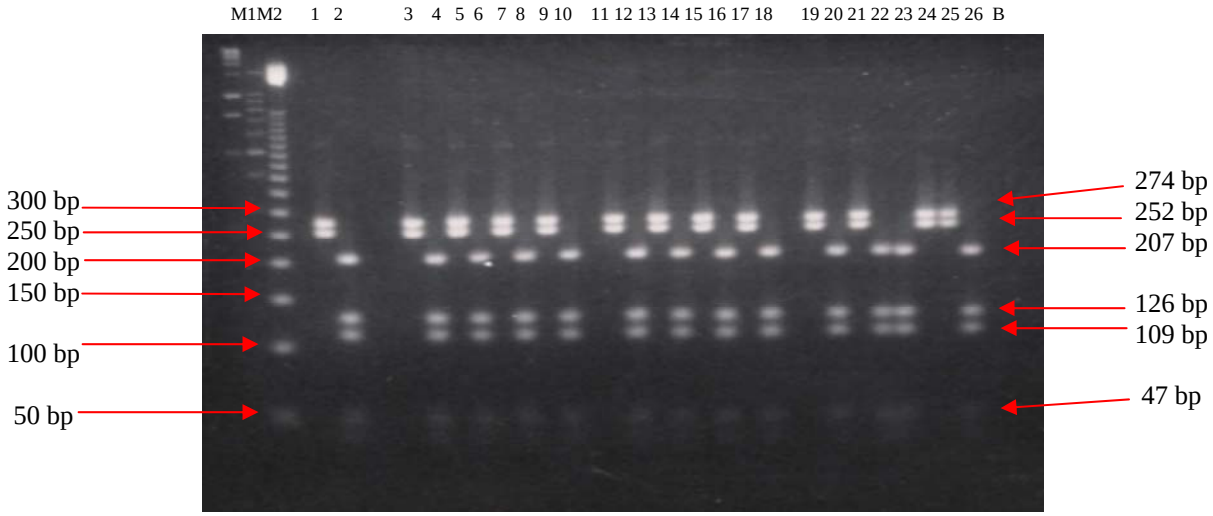


Figure 3.6: RFLP analysis of S-region amplified HBV clones on a 3% composite gel.

The resulting pattern is characteristic of genotype A. M1 = 100 bp molecular weight marker; M2 = 50 bp molecular weight marker; B = BQW blank. Lanes 1, 3, 5, 7, 9 = *Hinf* I restricted PCR products of RESP T2

Lanes 2, 4, 6, 8, 10 = *Tsp 509*I restricted PCR products of RESP T2

Lanes 11, 13, 15, 17, 19, 21, 24, 25 = *Hinf* I restricted PCR products of RESP T6

Lanes 12, 14, 16, 18, 20, 22, 23, 26 = *Tsp 509*I restricted PCR products of RESP T6

3.4 SUBGENOTYPING

3.4.1 A1 versus A2

To differentiate between subgenotypes A1 and A2 of genotype A, digestion with *Stu*I (Promega, Madison, WI) was carried out following the amplification of a 671bp amplicon (nucleotide position 521-1192) (Kew *et al*., 2005). All genotype A clones were found to belong to subgenotype A1 as demonstrated by 593 bp and 78 bp bands (*Figure 3.7*).

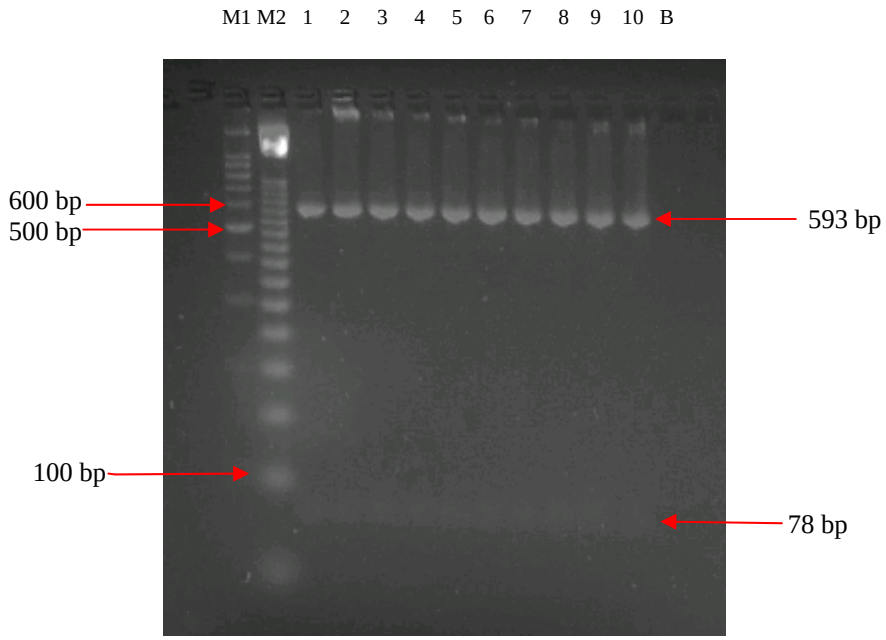


Figure 3.7: Subgenotyping of HBV clones on a 3% composite gel demonstrating the characteristic 593 bp and 78 bp bands unique to subgenotype A1. M1 = 100 bp molecular weight marker; M2 = 50 bp molecular weight; lanes 1-10: NON-RESP (T2) clones 1 -10; B = BQW blank.

3.5 DNA SEQUENCING AND ANALYSES

The DNA sequences of the preS1/preS2 and S genes were determined from serum samples taken from HBV infected Black children before, during and after IFN treatment (*Table 3.1*) and compared with corresponding sequences of HBV from GenBank. A total of 93 sequences of the surface gene of HBV were analysed in this study: 33 clones from a responder, 27 clones from a reverter and 33 clones from a non-responder. The alignments were edited manually by GeneDoc (Nicholas, 1997).

Table 3.1: Summary of the number of surface gene clones analysed in this study

Sample	**Number of clones analysed for each time point**				**Total number of**
	T0	**T2**	**T4**	**T6**	**clones**
RESP	10	10	5	8	33
REV	9	8	4	6	27
NON-RESP	10	10	3	10	33

Using the sequences from the clones, the nucleotide/amino acid divergences were calculated for each isolate at all time points.

3.5.1 Comparison of Mutations in a Responder, Reverter and Non-responder

In order to compare the HBV population in the responder, reverter and non-responder, DAMBE (Data Analysis in Molecular Biology and Evolution) (Xia, 2000) was utilized to calculate nucleotide divergences of clones compared to a reference sequence, AY233274 (GenBank accession number) (*Table 3.2*). Amino acid divergences were then calculated (*Table 3.3*) and together with nucleotide divergences, were expressed as means ± standard deviation as a percentage, in order to determine the variation within the samples at each time point. Using the GraphPad InStat program, one-way ANOVA's were performed on the data to test whether mean nucleotide and amino acid divergences differed amongst the responder, reverter and non-responder, and at the various times of sampling. When $p<0.05$, the variation was statistically different. The statistically significant differences in the nucleotide and amino acid divergences between the various times of sampling are highlighted in red (*Table 3.2 and 3.3*).

- In the case of the responder the only significant difference at the nucleotide and amino acid level was in the preS2 region between the time points. The nucleotide divergence between T0 and T4 was found to be statistically significant (*Table 3.2*), and related to a significant amino acid difference between T4 and all other time points (*Table 3.3*). Clones from T4 displayed the highest amino acid divergences.
- In the reverter a statistically significant difference in nucleotide divergence within the entire S gene was observed between T2 and all other time points of sampling

(*Table 3.2*), This was as a direct result of the significant differences in preS1 and HBsAg of the reverter (*Table 3.2*). HBV isolates sampled at T6 displayed the highest nucleotide divergence. However, when the amino acid divergence was compared, the significant difference was observed between T4 and all other time points of sampling (*Table 3.3*), with isolates from T4 showing the highest amino acid divergence. The amino acid variation in the entire S region of the reverter could be accounted for by the significant differences observed in the preS1 and preS2 region but not HBsAg.

- Interestingly the nucleotide variation in the case of the non-responder was only seen in the highly conserved HBsAg between T6 and all other time points (*Table 3.2*), with T6 showing a significantly higher nucleotide divergence than other time points. However, this significant difference in HBsAg did not carry through to the amino acid divergence indicating that the difference in nucleotide divergence was as a result of silent mutations. When looking at amino acid variation, a statistically significant difference was observed between time points in the preS1 region, with variation being the highest at T0 (*Table 3.3*).

Table 3.2: Mean nucleotide divergence (%) of the entire surface gene for a responder, reverter an non-responder before, during and after IFN treatment

Region	Sample	Nucleotide divergence and standard deviation*				P value¶
		T[0]	T[2]	T[4]	T[6]	
Entire S	RESP	2.29±0.21	2.45±0.18	2.38±0.13	2.27±0.23	0.2011
	REV	2.63±0.18	2.13±0.17	2.64±0.37	2.72±0.10	<0.0001
	NON-RESP	2.19±0.17	2.04±0.18	2.00±0.17	2.07±0.22	0.2577
PreS1	RESP	3.81±0.20	4.06±0.15	3.64±0.73	3.85±0.20	0.1142
	REV	4.08±0.37	2.98±0.21	3.99±0.35	3.83±0.14	<0.0001
	NON-RESP	3.05±0.21	2.83±0.38	2.71±0.43	2.83±0.31	0.2626
PreS2	RESP	2.85±0.29	3.27±0.42	4.24±1.42	3.33±0.92	0.0201
	REV	5.56±0.20	4.95±0.83	5.03±1.60	5.49±0.39	0.3391
	NON-RESP	4.48±0.42	4.30±0.19	4.24±0.00	4.12±0.85	0.5281
HBsAg	RESP	1.37±0.40	1.41±0.26	1.26±0.25	1.17±0.29	0.3995
	REV	1.17±0.24	1.01±0.28	1.36±0.18	1.47±0.19	0.0086
	NON-RESP	1.17±0.35	1.07±0.23	1.08±0.08	1.77±0.31	<0.0001

†Sequences compared to AY233274 (GenBank accession number)

*Nucleotide divergences ± standard deviations expressed as percentages

¶ This measures the significant difference between the time points within each patient

Table 3.3: Mean amino acid divergence (%) of the entire surface gene for a responder, reverter and non-responder before, during and after IFN treatment

Region	Sample	Amino acid divergence and standard deviation at various time points after treatment*				P value¶
		T[0]	T[2]	T[4]	T[6]	
Entire S	RESP	3.03±0.46	3.48±0.59	3.05±0.60	3.06±0.46	0.2089
	REV	3.03±0.36	2.91±0.35	3.63±0.32	2.92±0.30	0.0111
	NON-RESP	3.38±0.38	3.23±0.34	3.00±0.00	2.95±0.51	0.1138
PreS1	RESP	4.54±0.43	4.87±0.66	4.20±1.33	4.62±0.45	0.3749
	REV	3.17±1.09	2.73±0.74	4.83±1.06	3.36±0.00	0.0051
	NON-RESP	2.86±0.43	2.60±0.48	2.24±0.48	2.35±0.35	0.0453
PreS2	RESP	6.72±0.88	7.45±0.58	9.82±1.00	7.50±1.17	<0.0001
	REV	10.91±0.00	11.37±1.29	12.73±1.48	10.91±1.15	0.0407
	NON-RESP	11.09±1.03	10.91±0.00	10.91±0.00	10.36±1.73	0.5162
HBsAg	RESP	1.28±0.74	1.81±0.73	1.15±0.67	1.16±0.75	0.2055
	REV	0.98±0.53	0.77±0.31	1.00±0.67	1.18±0.36	0.4477
	NON-RESP	1.68±0.58	1.77±0.51	1.62±0.25	2.17±0.57	0.1762

†Sequences compared to AY233274 (GenBank accession number)

*Amino acid divergences ± standard deviations expressed as percentages.

¶ This measures the significant difference between the time points within each patient

Figure 3.8 compares the differences in amino acid divergences of the S gene and its regions between the strains from the responder, reverter and non-responder. Within the entire surface gene, no significant difference in amino acid divergence was observed between the responder, reverter and non-responder (*Figure 3.8A*). However, within preS1, the amino acid divergence of the strains obtained from the responder were significantly different from those of the non-responder ($p=0.0024$) (*Figure 3.8B,* blue versus yellow plot). The amino acid variation within the non-responder was found to be much lower than that of the responder throughout all the time points. It is also clear from figure 3.8B, that the reverter showed the greatest expansion of quasispecies over time compared with that of the responder and non-responder, where the population variation remained constant over time.

In the preS2 region the amino acid variation of the strains from the responder (blue plot) were significantly lower at all time points compared to the reverter ($p<0.01$) (pink plot) and non-responder ($p<0.01$) (yellow plot) (*Figure 3.8C*). Before initiation of treatment T0, the non-responder and reverter had a higher amino acid divergence compared with that of the responder. The same was true for T2 and T6. However, at T4 the amino acid variation within the reverter increased sharply. Overall, the responder showed the lowest change in amino acid variation, with the reverter showing the highest variation. The population within the non-responder remained relatively constant over time. In both the responder and reverter, treatment seemed to be controlling the dominant population but at T4, another population emerged, thus contributing to the expansion of the quasiespecies.

In the most conserved region, HBsAg, the only significant difference between amino acid divergences occurred between strains from the non-responder and reverter at all time points (p=0.038) (*Figure 3.8D*). Although amino acid variation stayed relatively constant over time for all three patients, it was lower in isolates from the reverter than in the isolates from the non-responder at all time points (*Figure 3.8D*, pink plot versus yellow plot).

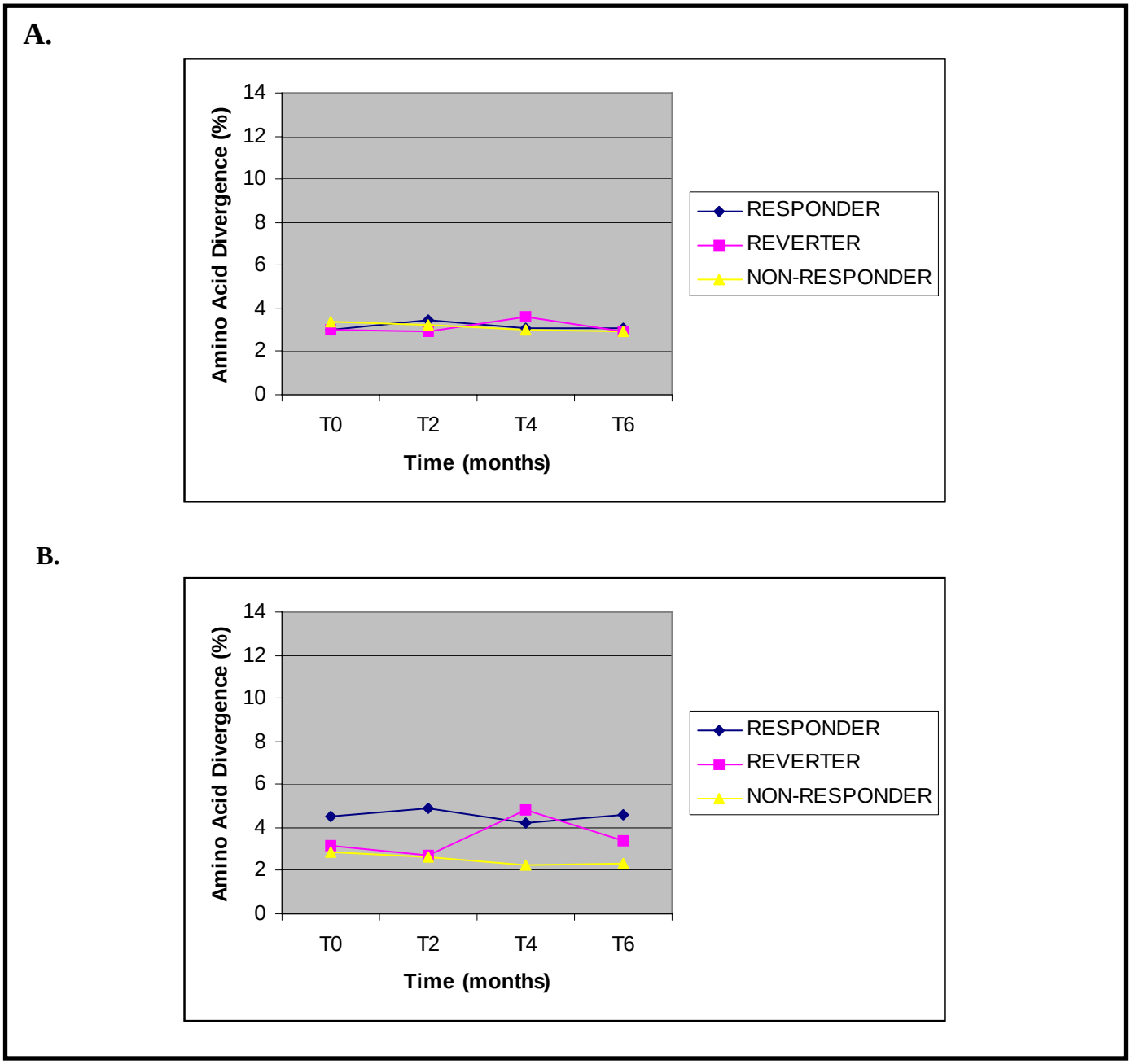
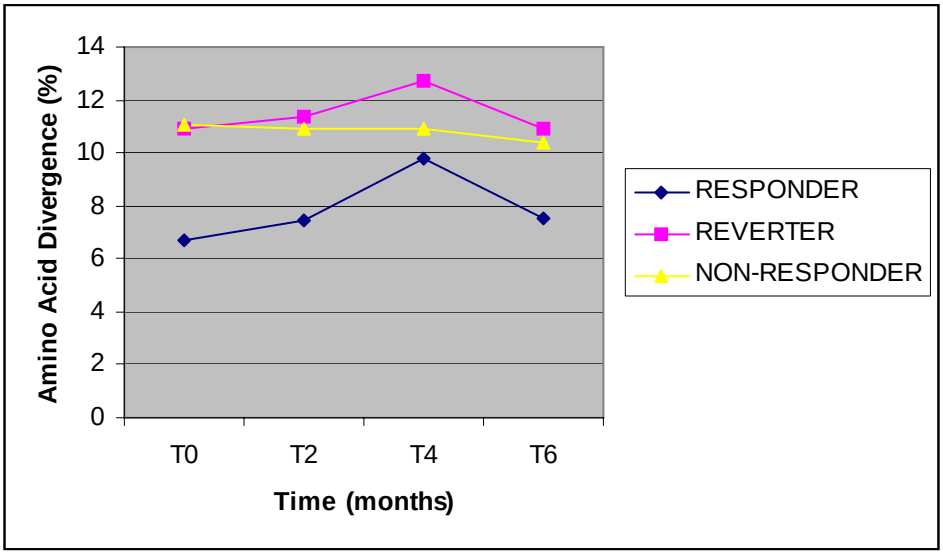


Figure 3.8: Comparison of amino acid divergences (%) over A.) the entire surface gene and B.) the preS1 region, for a responder, reverter and non-responder over time.

C.



D.

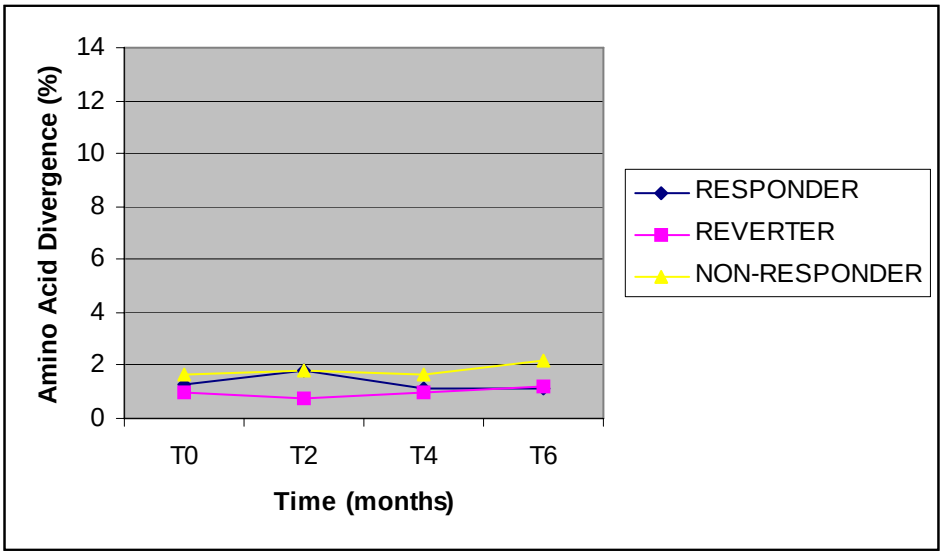


Figure 3.8: Comparison of amino acid divergences (%) over C.) the preS2 region and D.) HBsAg, for a responder, reverter and non-responder over time.

Figure 3.9 summarizes the results depicted in tables 3.2 and 3.3 and figure 3.8. For all patients, at all time points, the most variable region was found to be preS2, while HBsAg was the most conserved (*Figure 3.9*). When comparing the patients, the lowest variance within preS2 was seen in the responder with the highest being seen in the reverter (*Figure 3.9*). The lowest divergence within HBsAg was observed in the reverter, with the highest being in the non-responder (*Figure 3.9*). The amino acid divergence of S region as a whole, the preS1, preS2 and HBsAg of the strains from the non-responder remained constant over time (*Figure 3.9C*). The amino acid divergence of the responder and reverter however, varied between the different time points (*Figure 3.9A and B respectively*) demonstrating the emergence of new populations over the course of therapy.

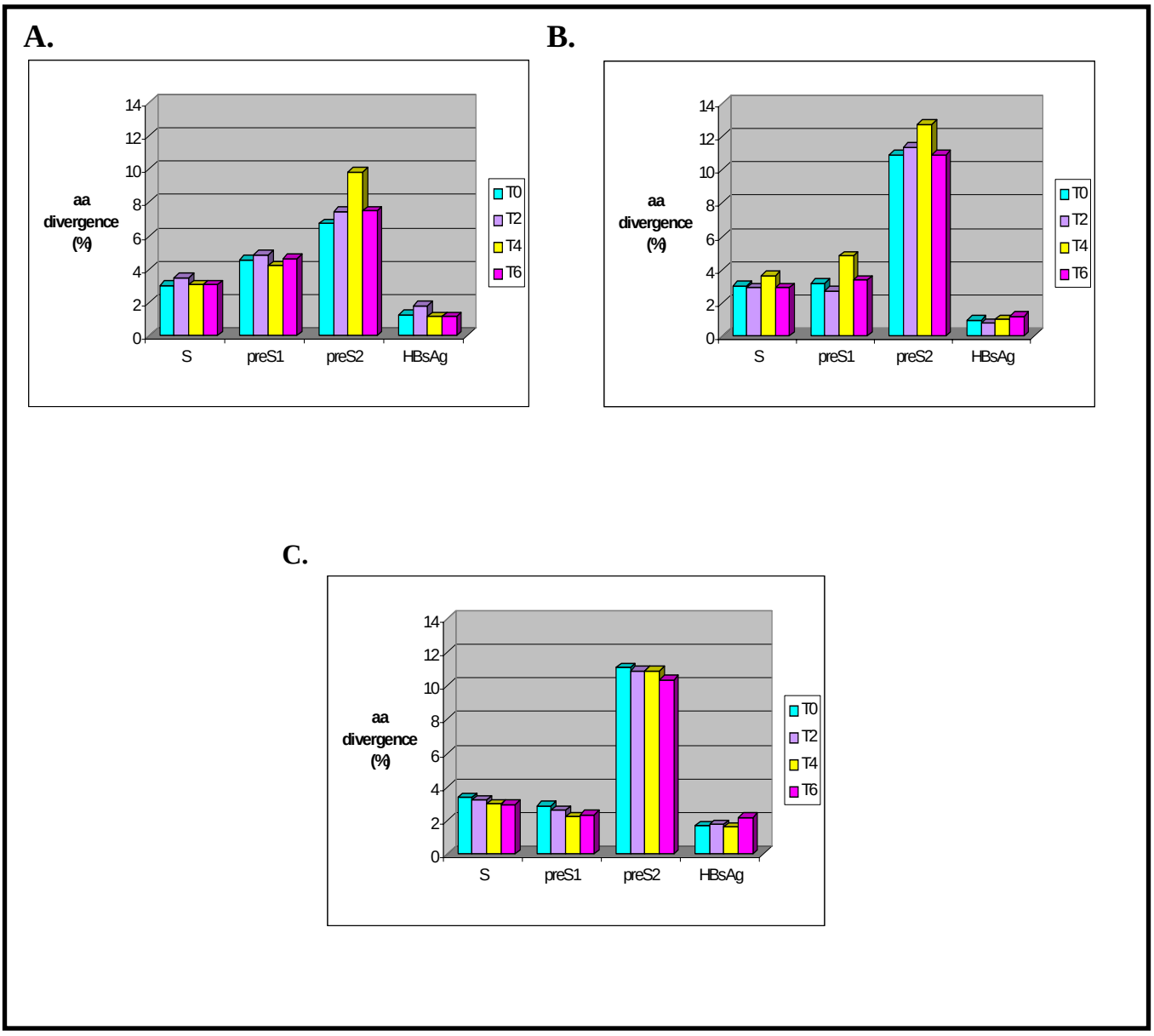


Figure 3.9: Comparison between amino acid divergences within the regions of the surface gene before, during and after IFN treatment. Amino acid divergence for A.) a responder B.) reverter and C.) a non-responder.

3.5.2 Analysis of Genetic Variability of the Surface Gene

Based on the amino acid composition of the HBsAg deduced from the nucleotide sequences, the serological subtypes of the clones of all isolates were determined to be *adw*2 (*Table 3.4*). One clone sequenced from the reverter at T4 had a Lys^{160} to Glu^{160} and Phe^{134} to Ile^{143} change, which did not however affect the serological subtype.

Table 3.4: Amino acid residues in HBsAg involved in *adw*2 subtype determination

Subtype	**aa 122**	**aa 125**	**aa 127**	**aa 134**	**aa 143**	**aa 159**	**aa 160**	**aa 168**
*adw*2	Lys	Thr	Pro	Phe	Thr	Ala	Lys	Val

Various amino acid changes were observed in the responder, reverter and non-responder in comparison to the reference sequence used namely AY233274 (GenBank accession number). Only those mutations occurring in either more than two clones or in two or more of the isolates will be discussed.

The presence of unique amino acid (aa) residues in the preS1 and preS2 region of HBV, characteristic of subgenotype A1, were found in all clones of the non-responder, confirming previous subgenotyping RFLP analyses, which showed the non-responder to belong to subgenotype A1 (*Section 3.4.1*). The unique amino acids in the preS1 region were: Gln^{54}, Val^{74}, Ala^{86} and Val^{91} and in the preS2 region: Leu^{32}.

In the responder the most frequently observed mutation in the preS1, was an A-to-T transversion at nucleotide (nt) 3009, which resulted in a Gln^{54} to His^{54} substitution. Interestingly, this mutation, occurring within a subgenotype determinant residue, was observed in 100% of clones from T0 and T6 and in 90% and 60% of clones from T2 and T4 respectively (*Table 3.5*) and may account for the fact that the responder showed the highest amino acid divergence within preS1, compared with the non-responder and reverter at T0, T2 and T6. Another mutation, a Val^{91} to Phe^{91}, was found in 50% of the T2 clones from the reverter (*Table 3.5*). Both the aforementioned amino acid changes were not characteristic of any other genotype and are thus unique and do not appear to affect the subgenotype of the strains as demonstrated by the fact that the RFLP pattern did not change.

Leu^{32}, which has been identified as being characteristic of subgenotype A1 (Kimbi *et al.*, 2004) was mutated to a His^{32} in all clones of the responder at all time points and in 50% of clones from T2 and T4 of the reverter. Within the major antigenic epitope region of preS2, a Gln^{10} to Lys^{10} change was found in 100% of the clones from the non-responder at T0, T2, and T4 and in 90% of clones from T6 (*Table 3.5*). A different mutation at this position was also observed in strains from the responder and reverter. A Gln^{10} to Arg^{10} substitution was present in 40% of clones from the responder at T4 as well as in 100% of T0 and T6 clones, 37.5% of T2 clones and 50% of T4 clones from the reverter (*Table 3.5 and Figure 3.10*). Interestingly, in the preS2 region, the amino acid divergence of the non-responder remained constant throughout treatment, whereas the amino acid

divergence of the responder and reverter varied, becoming especially high at T4. The Gln^{10} to Arg^{10} mutation may possibly contribute to this trend.

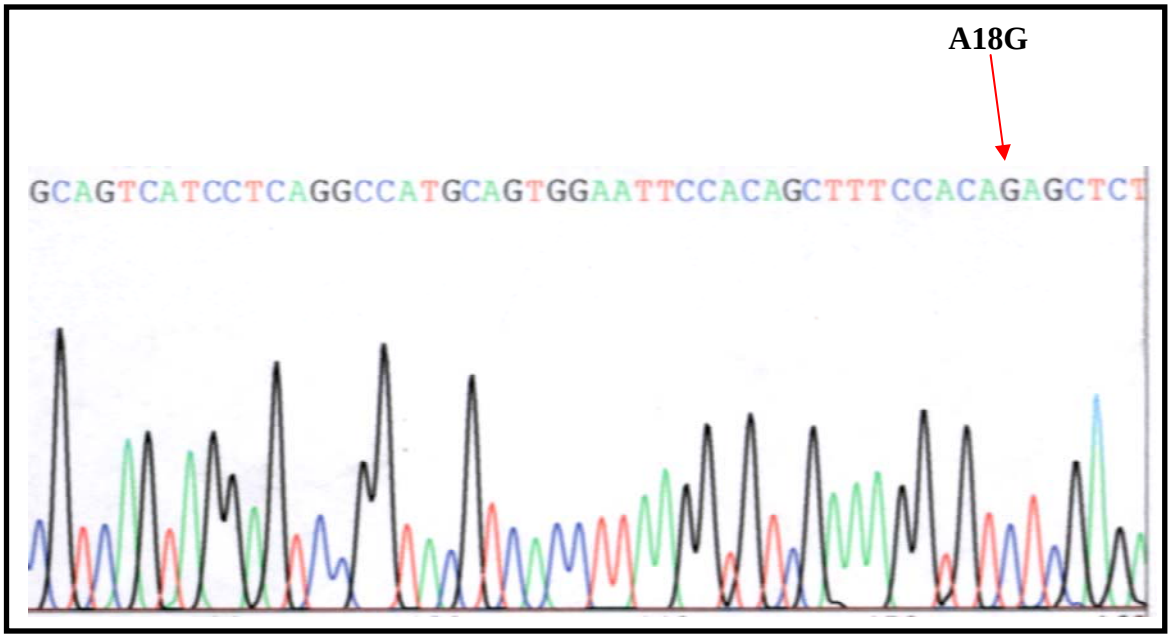


Figure 3.10: A chromatogram demonstrating the Gln^{10} to Arg^{10} mutation found in the responder and reverter as a result of an A-to-G substitution at nt 18 of the preS2 region.

Variation in the amino acid sequence was found throughout the small envelope protein (*Figure 3.11*). A Met^{1} to Val^{1} substitution was seen in 20% of clones from the responder at T0 and a Met^{1} to Thr^{1} mutation was observed in 10% of clones from the non-responder at T2 (*Table 3.5*). Both these mutations occurred within the ATG start codon and would probably affect the synthesis of the HBsAg.

Two sets of mutations were detected just upstream of helix I of HBsAg, on the outside of the ER membrane. A Glu^{2} to Gly^{2} substitution, was found in 50% of clones from the reverter at T2 and a Phe^{8} to Ser^{8} change in 20% of clones from the responder at T2 and in 10% of clones from the non-responder at T0 (*Table 3.5*). Within 50% of clones from the reverter at T2, a Leu^{13} to Phe^{13} mutation was found in helix I of HBsAg (aa 11-29), which crosses the ER membrane and translocates the upstream sequence into the ER lumen (*Table 3.5*). In the major cytoplasmic loop of HBsAg, two mutations were detected in the non-responder: a Leu^{49} to Arg^{49} in 8 clones; 20% of clones from T2 and 60% from T6 and Leu^{77} to Arg^{77} substitution occurred in 20% of clones from T0 and 10% of clones from T2 (*Table 3.5*).

Mutations were also present within the second helix of HBsAg corresponding to the immunodominant region (aa 101-168) or major hydrophilic region (MHR). A Met^{103} to Ile^{103} substitution was present in the 25% of T4 clones and 50% of T6 clones from the reverter (*Table 3.5*). Downstream of this, a Gly^{112} to a Arg^{112} mutation occurred in 3 clones from the reverter; two from T2 (25%) and one from T4 (25%) and in five clones from the non-responder; four from T2 (40%) and one from T4 (33%) (*Table 3.5*).

The clones from the responder and reverter both contained mutations in the major antigenic determinant of HBsAg, the ‘a’ determinant (aa 124 to 147), thus possibly having a pronounced effect on antigenicity. A Gln^{129} was substituted for an Arg^{129} in one clone from each time point, T0 (11%), T2 (12.5%) and T6 (17%) of the reverter (*Table 3.5*). Further down, a Met^{133} to a Thr^{133} mutation was seen in 25% of clones from the

reverter at T4 and occurred in 40% of T0, 50% of T2, 60% of T4 and 50% of T6 clones from the responder (*Table 3.5*). Still within the 'a' determinant, a Asp^{144} to Gly^{144} mutation, found in the responder at all time points and a Asp^{144} to Ala^{144} occurring in one clone of the non-responder at T4, was found as a result of a A-to-G or a A-to-C transition at nt 585 (*Table 3.5*). A Glu^{164} to Gly^{164} substitution was present in 20% of clones at T0, 50% of clones at T2 and 12.5% of clones at T6 of the responder as well as in 33% of clones from T0 and in 17% of clones from T6, of the reverter (*Table 3.5*).

Two further mutations were identified. A G-to-A transition at nt 700 led to a Trp being substituted for a stop codon at aa 182 in the non-responding patient in 10% of clones from T0, 20% of clones from T2 and 67% of clones from T4 (*Table 3.5*). This mutation would possibly lead to a truncated HBsAg protein and have a pronounced effect on patient response. Also, in the responder, a Leu^{186} was substituted for a Pro^{186} in 20% of T0 and T2 and 37.5% of T6 clones (*Table 3.5*). Although these mutations were found outside of the major antigenic determinant downstream of helix II, they could nevertheless still affect antigenicity.

Table 3.5: Summary of unique mutations present within the surface gene of IFN-treated patients

HBV Region	Nucleotide* Substitution	Amino acid Substitution	Sample	Number of clones with mutation	% of clones with mutation
PreS1	A3009T	Q54H	RESP	29/33	88%
	G3118T	V91F	REV	2/27	7%
PreS2	C17A	Q10K	NON-RESP	32/33	97%
	A10G	Q10R	REV	20/27	74%
			RESP	2/33	6%
HBsAg	A155G	M1V	RESP	2/33	6%
	T156C	M1T	NON-RESP	1/33	3%
	A159G	E2G	REV	4/27	15%
	T177C	F8S	NON-RESP	1/33	3%
			RESP	2/33	6%
	C191T	L13F	REV	4/27	15%
	T300G	L49R	NON-RESP	8/33	24%
	T384G	L77R	NON-RESP	3/33	9%
	G463C	M103I	REV	4/27	15%
	G488A	G112R	NON-RESP	5/33	15%
			REV	3/27	11%
	A540G	Q129R	REV	3/27	11%
	T552C	M133T	REV	1/27	4%
			RESP	16/33	48%
	A585C	D144A	NON-RESP	1/33	3%
	A585G	D144G	RESP	15/33	45%
	A645G	E164G	REV	4/27	15%
			RESP	8/33	24%
	G700A	W182STOP	NON-RESP	5/33	15%
	T711C	L186P	RESP	7/33	21%

* Sequences compared to AY233274 (GenBank accession number), where position 1 corresponds to the *EcoR*I cleavage site.

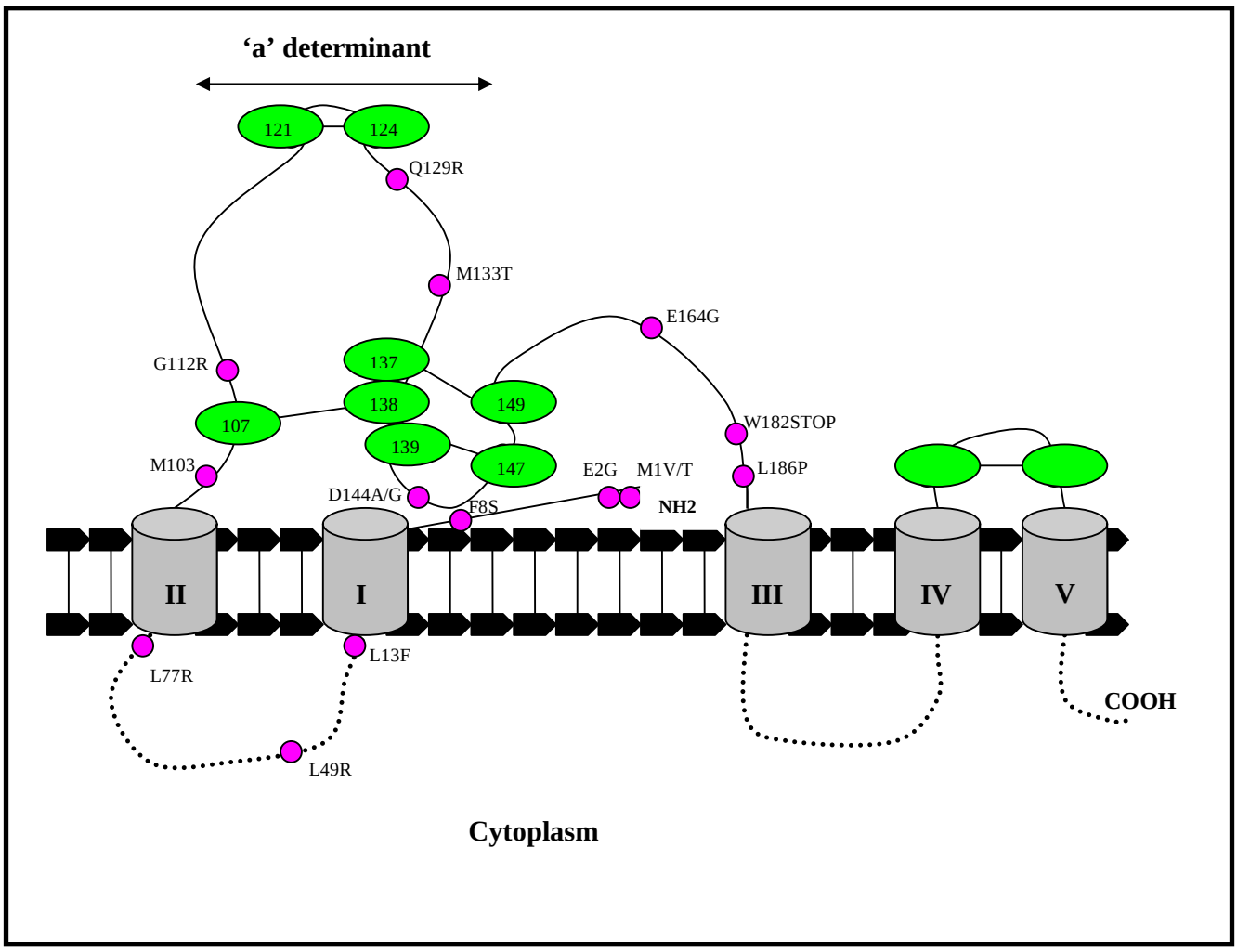


Figure 3.11: Graphic representation of mutations found within the small envelope protein of the responder, reverter and non-responder clones.

3.6 PHYLOGENETIC ANALYSIS

DNADIST was used consecutively with NEIGHBOR (neighbour-joining) to generate dendograms of all surface gene clones from the responder, reverter and non-responder at all time points. Trees were then visualized using TreeView Win 32 software program (Page, 1996) together with isolates corresponding to all genotypes from GenBank in order to access the genotype distribution of these samples. Analysis of samples showed the clustering of the responder, reverter and non-responder with genotype A1, confirming previous analysis. There was no separation of the strains in each patient according to time of sampling showing that within each patient there was a mixed population.

Figure 3.12 demonstrates the phylogenetic trees visualized using TreeView Win 32 software program (Page, 1996) for the responder, reverter and non-responder at the different times of sampling:

- At T0, the patients form distinct clusters, with strains from each patient clustering together. The strains of the reverter and non-responder were the most similar sharing the same root and clustering together.
- At T2, the responder and the non-responder keep the same cluster as at T0, showing that the population remains the same in these patients. However, the reverter separates into two clusters with separate roots, demonstrating the emergence of an additional population.
- At T4, the responder clustered into two distinct populations. Within the strains of the reverter, a completely new population emerged as shown by the two distinct

clusters, even more separate from those at T2. In the non-responder, a new population also emerged distinct from that at T0 and T2.

- At T6, the new populations within the responder, reverter and non-responder were knocked out causing the original populations present at T0 to re-emerge, regardless of whether the patients were a responder, reverter or non-responder.

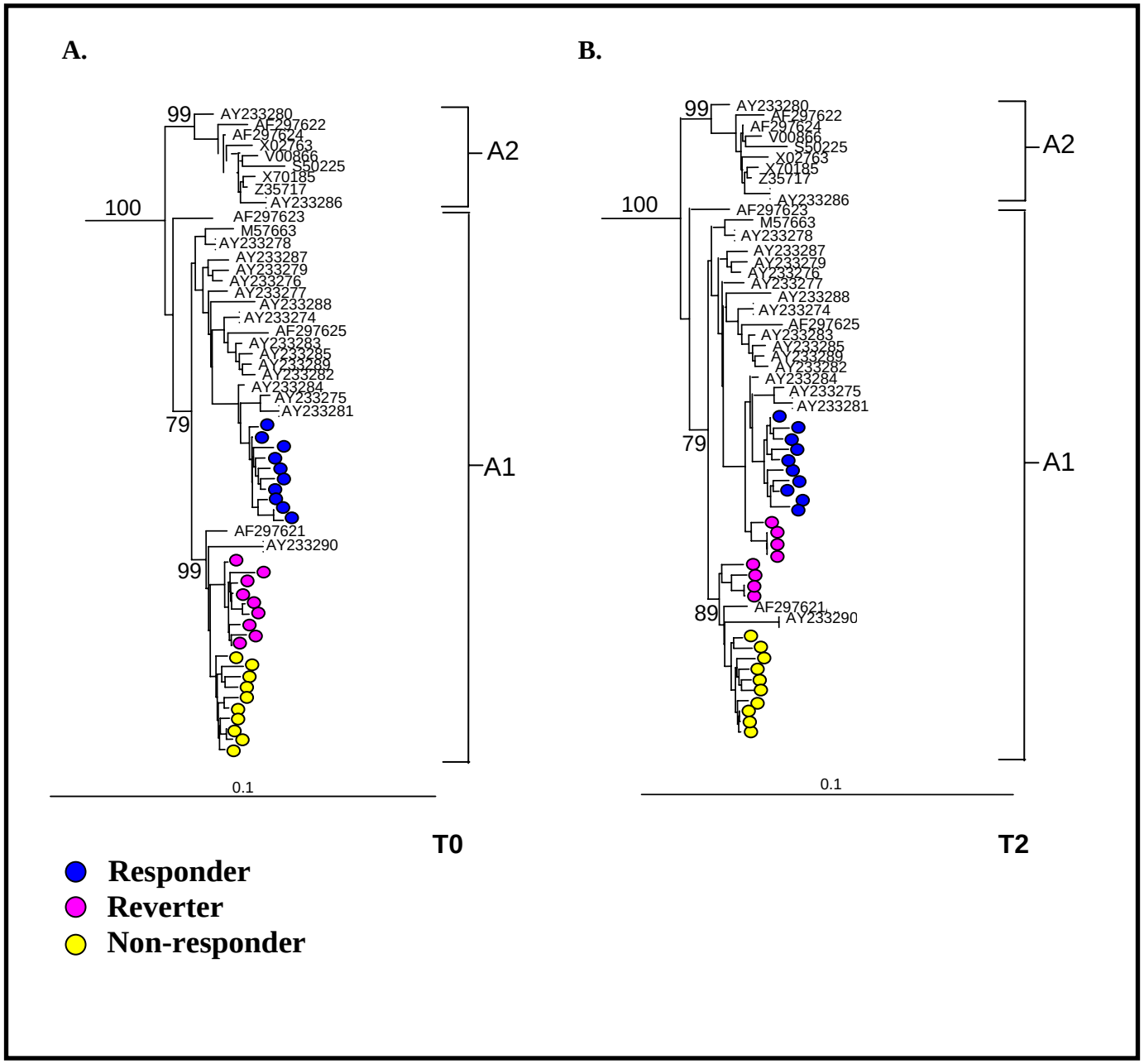


Figure 3.12: Phylogenetic tree comparing the complete S ORF of the responder, reverter and non-responder at A.) T0 and B.) T2 to other genotype A HBV isolates obtained from GenBank (designated by its accession number) established using neighbor-joining. Bootstrap statistical analysis was performed using 1 000 data sets and the numbers on the nodes indicate the percentage of occurences.

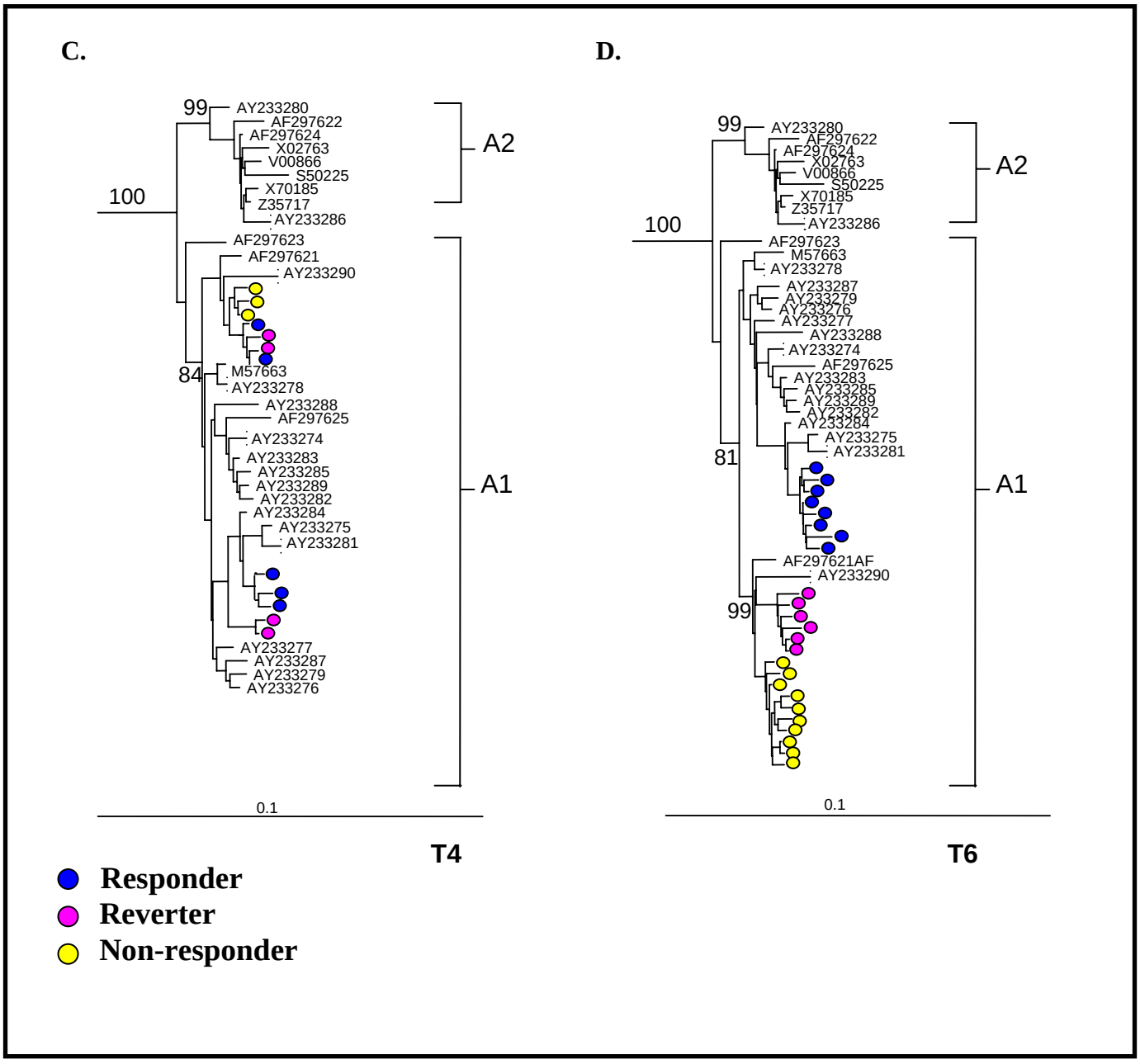


Figure 3.12: Phylogenetic tree comparing the complete S ORF of the responder, reverter and non-responder at C.) T4 and D.) T6 to other genotype A HBV isolates obtained from GenBank (designated by its accession number) established using neighbor-joining. Bootstrap statistical analysis was performed using 1 000 data sets and the numbers on the nodes indicate the percentage of occurences.

Chapter 4: DISCUSSION

A number of drugs have been used in the treatment of HBV infection, including interferon. The response to IFN is usually measured by HBe seroconversion, reduction of viraemia, alanine aminotransferase levels and histological examination (Lindh, 1997). The ability of IFN to enhance the rate of viral clearance, leading to loss of HBeAg, however, only extends to 30-40% of treated patients. The reason for this is unclear. Viral sequence heterogeneity may be an important predictor of IFN response. Although a number of studies have been carried out to determine the influence of viral sequence heterogeneity on IFN response (Takeda *et al.*, 1990; Zampino *et al*., 2002; Chen *et al*., 2003), none have been within the South African context. Considering that the majority of South African HBV isolates belong to subgenotype A1 and have unique sequence characteristics (Kimbi *et al*., 2004), studies on patient response from other countries cannot necessarily be directly extrapolated to studies on South African patients. Moreover, the genotype of the virus can have important implications in the development of mutations and response to therapy (Kramvis and Kew, 2005).

After analysis of the catalytic domains of the polymerase gene, the BCP and precore and core regions of HBV following IFN treatment, Chen *et al* (2003) concluded that the basis of non-response or relapse did not lie in these regions and thus must reside in other parts of the HBV genome. The surface gene may therefore be a suitable candidate for predicting patient response to IFN. Not only does the pre-S sequence contain the highest heterogeneity over the entire genome (Lauder *et al*., 1993; Günther *et al*., 1999), but the

preS2 region in particular, is known to be hypervariable (Huy *et al*., 2003). The ability to predict response to IFN would have profound implications in the prognostic and therapeutic arena. Therefore, the aim of the present study was to determine whether the sequence of preS1/preS2/S region could be used as a predictive factor for IFN response in southern African Black carriers of the virus.

Sequencing of the various open reading frames of HBV from subgenomic amplicons does not allow the analysis of the mutations present in a single molecule of the complete HBV genome (Günther *et al*., 1995). Therefore, in this study, we attempted to amplify the complete genome of HBV isolates from a responder, reverter and non-responder, at various times post-treatment. This complete genome, however, could only be amplified when the HBV DNA levels were high as was the case before treatment for all patients, at all time points for the non-responder and when the levels of HBV were at pretreatment levels in the case of the reverter. Therefore we used subgenomic PCR of the complete S ORF for the responder and reverter.

In infected individuals, HBV exists as a whole population of phylogenetically related variants or quasispecies (Smith *et al.*, 1997), with one strain dominating. In order to maintain the stability of the pool, certain selection pressures from the host's immune system, constraints imposed upon by the reading frames, and the viability and replication competence of the virus need to remain intact (Locarnini *et al*., 2003). Thus the interaction between viral and host factors as well as external pressures define the dominant HBV population (Locarnini *et al*., 2003). However, with the introduction of

IFN treatment, the balance may be disrupted, leading to the emergence of mutant HBV strains or genotypes able to withstand therapy and consequently causing a non-responsive outcome. In order to identify these minority strains or genotypes, the amplicons were cloned and the clones genotyped/subgenotyped and sequenced.

The genotype of HBV has been shown to influence the response to IFN therapy (Kramvis and Kew, 2005) and when patients treated with interferon contain genotype mixtures (Hannoun *et al*., 2002b), genotype switches can occur (Gerner *et al*., 1998). Genotypes A and D have been shown to occur in the southern African Black population (Bowyer *et al*., 1997; Kimbi *et al*., 2004), with the unique segment of genotype A, subgenotype A1 predominating (Kimbi *et al*., 2004). To determine whether the genotype/subgenotype may influence response to IFN treatment, the clones of the HBV isolates were genotyped/subgenotyped. All HBV clones, at all time points, regardless of IFN response of the patient, belonged to subgenotype A1 of genotype A as shown using RFLP analysis (*Figures 3.6 and 3.7*) and had the distinctive sequence characteristics of this subgenotype. All isolates had serological subtype of *adw*2. Therefore differences in response to IFN treatment were not as a consequence of genotype or serotype diversity of the HBV isolates obtained from the different patients.

The next step was to sequence the preS1/preS2/S region of the clones of the isolates, to establish whether sequence heterogeneity in this region of the HBV genome could account for differences in the response to IFN treatment. Quasispecies have been implicated in failure of vaccines and therapies (Domingo, 1998) and in the incorrect

diagnosis of infectious diseases caused by viruses such as HIV (Brenner *et al*., 2002) and HCV (Gonzalez-Peralta *et al*., 1996) that are characterized by error prone replication. Although HBV is not a RNA virus, it replicates by reverse transcription of the pregenomic RNA using a polymerase that lacks proofreading ability and thus sequence heterogeneity and the evolution of a quasispecies is also a feature of this virus (Steinhauer and Holland, 1986).

When determining sequence heterogeneity, it is important, as cautioned by Smith and coworkers (1997), to minimize the number of artefactual substitutions introduced by PCR amplification of the virus sequences (Smith *et al*., 1997). To do this, in the present study, we took the following precautions:

- We used Expand High Fidelity Enzyme mix that combines *Taq* DNA polymerase, a high amplification efficiency enzyme, with *Tgo* DNA polymerase, a thermostable enzyme with a low error rate and proofreading activity.
- Because of the high concentration of HBV DNA following serum extraction in some samples, the DNA extracts were diluted to 1:2 or 1:5 in order to achieve efficient amplification and to prevent the saturation point of amplification being reached early and leading to the incorporation of artefactual mutations.
- The annealing temperature for amplification was optimized for each individual sample in order to minimize mispriming. Although Günther utilized an annealing temperature of 60ºC, in this study annealing temperatures of either 57º or 60ºC at a 1:2 dilution were found to be the most effective for full genome amplification.

- In order to differentiate between artefactual and genuine substitutions, sporadic substitutions (i.e. occurring in only one of the multiple clones sequenced) were not taken into account when calculating nucleotide divergences.
- Moreover, from the sequences of the S ORF of the clones the nucleotide divergences were calculated (*Table 3.2*) and the error rate per nucleotide per PCR cycle was calculated to be between 2.5 and 3.4 x 10^{-4}, which is much higher than the expected error rate of *Taq* polymerase (0.2 – 2 x 10^{-4}) (Smith *et al*., 1997).

When summarizing the results obtained for amino acid divergences over all the regions of the S ORF for the different patients (*Figure 3.9*), in agreement with other studies (Bowyer and Sim, 2000; Lauder *et al*., 1993), it was clear that the preS2 region was the most variable for all patients and HBsAg was the most conserved. This is to be expected because the preS2 protein is not essential for HBV replication, virion morphogenesis, or secretion, and is not required for infectivity (Fernholz *et al*., 1993) whereas HBsAg has an essential role in the viral life cycle (Bruss and Ganem, 1991). However, because of the importance of the preS region in immunity and protection (D'Mello *et al*., 1997), the high viral variation in this region in the HBV strains isolated from all patients, may have an important effect on IFN response. The highest divergence in preS2 was seen in the reverter and non-responder, with the highest divergence in HBsAg being seen in the non-responder (*Figure 3.9C*). Again, this seems to highlight the fact that the high amino acid divergence in these regions may be as a result of certain mutations responsible for persistence of the virus and the absence of response to IFN. The significant increase in the amino acid divergence in the preS2 and HBsAg in the non-responsive patients

compared with that of the responder could indicate that mutations in these regions may carry predictive markers of non-response. Further investigation is therefore warranted.

Phylogenetic analysis of the clones confirmed the results of the RFLP analysis which showed all clones of HBV from all patients to cluster with subgenotype A1 within genotype A. Moreover, the trees (*Figure 3.12*) confirmed the relationships and population dynamics between the responder, reverter and non-responder reflected by the nucleotide (*Table 3.2*) and amino acid (*Table 3.3*) variations. When looking at the phylogenetic relationship between the patients at each time point, we see that at T0, the strains of each patient group together under distinct clusters, with the reverter and non-responder being genetically the most similar (*Figure 3.12A*). However, at T2 (*Figure 3.12B*), we see the separation of the strains within the reverter into two clusters, showing the breakthrough of a new population separate from the initial viral strains present at T0. IFN treatment therefore seems to be altering the balance within the HBV quasispecies pool and selecting for a mutant population able to withstand the treatment. The strains within the responder and non-responder remained the same as those at T0. Thus, the onset of quasispecies evolution was delayed in these patients. At T4 (*Figure 3.12C*), the responder showed two completely distinct clusters from those seen at T0 and T2 demonstrating the expansion of the quasispecies. The reverter showed further separation of its two distinct clusters, which was as a result of the expansion of the two populations seen at T2. Although the strains of the non-responder remained together, as a whole they clustered differently to the populations at T0 and T2. Thus, all three patients seem to demonstrate a heterogeneous population, able to mould itself according the selective pressure applied by

the immune system as a result of IFN therapy. IFN treatment is known to increase the host immune elimination pressure on HBV-infected cells and to result in the selection of HBV quasispecies (Chen *et al*., 2003). At T6 (*Figure 3.12D*), there was a reforming of the initial clusters in all patients, and thus a reversion to the initial major variant present at T0. Hence, in the patients, although there was accelerated quasispecies evolution during IFN treatment, the newly emerged populations disappeared at T6 after the removal of IFN, causing the clusters to regroup into the original strains found at T0.

In the responder, the most common preS1 mutation, occurring within a subgenotype determinant residue, was a Gln^{54} to a His^{54} substitution in 100% of T0, 90% of T2, 60% of T4 and 100% of T6 clones. This mutation was found in the original cluster of HBV isolated from the responder. Not characteristic of any other genotype (Kimbi *et al*., 2004), this mutation appears to be unique. Replacing glutamine, a polar amide with high flexibility with histidine, a polar, positively charged amino acid containing an imidazole ring, would not only change the flexibility of the protein at this point but also potentially alter the hydrophobicity of the amino acid. This mutation would possibly have an effect on the antigenicity of the protein and may differentiate the HBV isolates from the patient responding to IFN treatment from the non-responders. This mutation has been described in asymptomatic carriers with low viral loads (Xu *et al*., 2006)

Another unique mutation within a subgenotype determinant residue of preS1 occurred in two clones from T2 of the reverter. Substitution of a Val^{91}, a non-polar, hydrophobic unreactive amino acid for a Phe^{91} that, although also hydrophobic, is a globular amino

acid, would likely alter the tertiary structure of the preS1 region. As amino acids 81-108 of preS1 code for the T-cell epitopes and are therefore highly immunogenic (Chisari and Ferrari, 1995), a mutation of this nature would likely change the antigenicity of this region leading to failure of the T-cells to bind and neutralize HBV. However, as this mutation was only present at T2 of the reverter, it is unlikely that it had an effect and it was probably eliminated by IFN.

Although the preS2 region of HBV is hypervariable, mutations in this domain may modify the course of HBV infection (Huy *et al*., 2003). A Leu to His substitution at amino acid 32 of preS2 was found in 50% of clones from T2 and T4 of the reverter. Amino acid 32 is a unique subgenotype determinant of subgenotype A1 (Kimbi *et al*., 2004) and in the majority of isolates is characterized by a leucine. However, small minorities of A1 isolates contain a His^{32}. Thus, this mutation did not affect the subgenotype of the reverter, which was concluded to be A1 (Kimbi *et al*., 2004).

Within the major antigenic epitope region of preS2, known to carry numerous B-cell, T-helper cell and CTL epitopes (Sominskaya *et al*., 2002), a Gln^{10} to Lys^{10} substitution was found in 100% of clones from the non-responder at T0, T2 and T4 and in 90% of clones from T6. Similarly, a Gln^{10} to Arg^{10} mutation (*Figure 3.10*) was present in 40% of T4 clones from the responder and 100% of T0 and T6, 37.5% of T2 and 50% of T6 clones from the reverter. Glutamine, an uncharged amino acid being replaced by a lysine or an arginine, both positive polar amino acids, would likely affect the tertiary structure of the epitope. Arginine especially, is capable of intense molecular interactions due to a unique

moiety terminating its side chain, a guanidine group. Given that both these mutations lie within a T helper cell epitope region (amino acids 1-15), (Ong *et al*., 2005), altering the structure of this epitope would greatly affect the binding of the T-cells. As the Gln^{10} to Arg^{10} and Gln^{10} to Lys^{10} mutations were found throughout all time points of the reverter and non-responder, these mutations may be reducing the ability of the IFN-induced antibodies to bind to the epitopes and initiate their neutralizing effect (Wallace *et al*., 1997). Thus, the mutation may be providing a survival advantage within the IFN environment leading to non-response in these patients. The presence of this mutation may be playing a significant role in patient response to IFN and could potentially be used as a predictive marker for treatment outcome.

Within the ATG start codon of the HBsAg, a Met^{1} to Val^{1} mutation was observed in 20% of clones from the responder at T0 while the non-responder possessed a Met^{1} to Thr^{1} mutation in 10% of clones from T2. Both mutations appeared to be in the minority population at the start of treatment and were eliminated by T4, intimating that their inability to code for complete HBsAg may be a disadvantage to the survival of the strains.

Situated between helix I and II, within the cytoplasmic loop, two mutations were detected in the non-responder; a Leu^{49} to Arg^{49} mutation and a Leu^{77} to Arg^{77} mutation. Both would lead to a non-polar, unreactive, hydrophobic amino acid being substituted for a polar, positively charged, hydrophilic amino acid. Biochemically, this would more than likely affect the tertiary structure of the major cytoplasmic loop. However, with the case

of Leu^{77} to Arg^{77}, this seems improbable, as this mutation was only present in 20% of clones from T0 and 10% of clones from T2, showing a decrease in this mutation over time. With regards to the Leu^{49} to Arg^{49} mutation, it was present at the beginning of treatment at T2, in 20% of clones, disappeared at T4 and then reemerged at T6 in 60% of clones from the non-responder. Thus, it seems that the immune pressure applied by IFN was selecting for this mutant strain, possibly because it was resistant to treatment. As amino acids 29-80 of the cytoplasmic loop are involved in the interaction of the core particles with the envelope protein (Khan *et al*., 2004), a mutation in this region may also affect the encapsidation of the core particles after virion formation.

The MHR of HBsAg spanning amino acids 101 to 168, is orientated on the surface of the virus. 25% of T4 clones and 50% of T6 clones from the reverter possessed a Met^{103} to Ile^{103} mutation. Given the similar nature of these amino acids, both non-polar, unreactive and hydrophobic, the effect on the tertiary structure of the protein at this point seems insignificant. However, a Gly^{112} to Arg^{112} amino acid substitution would cause a non-polar, hydrophobic amino acid to be substituted for a polar, hydrophilic amino acid. Given the importance of this region and the fact that glycine is a very flexible amino acid, this mutation may not only affect the structural integrity of the protein, but may also affect the entire immunodominant region. Occurring in clones of the reverter and non-responder at T2 and T4, this mutation probably developed as a result of this highly immunogenic region being under selective pressure from the immune system (Mimms, 1995) and consequently may be playing a role in the establishment of resistance to IFN.

The major antigenic determinant of HBsAg, the 'a' determinant, spans amino acids 124-147 within the MHR (*Figure 3.11*) and plays an essential role in antigenicity. A commonly occurring vaccine-escape mutant, namely a Gln^{129} to His^{129}, has been found in this region (Wallace *et al*., 1997). A variation of this mutation corresponding to a Gln^{129} to Arg^{129} substitution was found in the reverter in one clone each from T0, T2 and T6, leading to a non-polar, hydrophobic amino acid being replaced by a polar hydrophilic amino acid. Khattab *et al* (2005) also found this mutation in the 'a' determinant of occult HBV in patients undergoing IFN therapy. A mutation in this region may significantly alter the conformation of the epitope thus affecting the binding of the hepatitis B surface antibodies (anti-HB's) to the 'a' determinant and reducing the antigenic effect (Khattab *et al.,* 2005).

Also within the 'a' determinant, 40% of T0 clones, 50% of T2 clones, 60% of T4 clones and 50% of T6 clones from the responder and 25% of the clones at T4 of the reverter possessed a Met^{133} to Thr^{133} point mutation. This led to a non-polar, hydrophobic amino acid being replaced by a polar, hydrophilic one, not only having an effect on the tertiary structure of the protein, but also affecting the hydrophibicity. Functional studies have shown enhanced HBsAg secretion and virion secretion associated with this mutation (Khan *et al*., 2004). Therefore the increased "fitness" may explain the survival and persistence of HBV in both the responder and reverter following IFN treatment.

Another commonly described vaccine escape mutant, Asp^{144} to an Ala^{144} (Wallace *et al*., 1997) occurred in one clone from the non-responder at T4. Even though this mutant is

known to evade the neutralizing effect of antibodies because of reduced antibody binding activity (Torresi, 2002), it is unlikely that it had any effect in this patient since it occurred at such low levels. Another mutation, Asp^{144} to Gly^{144}, which may represent a novel vaccine-escape mutant, occurred in the responder at all time points; 40% of T0 clones, 50% of T2 clones, 20% of T4 clones and 62.5% of T6 clones.

Lamivudine escape mutants such as Glu^{164} to Asp^{164}/Ile^{195} to Met^{195}, commonly occur as a result of mutations in the 'a' determinant, and may have the potential to become vaccine escape mutants (Torresi, 2002). A different mutation, namely a Glu^{164} to Gly^{164} mutation was present in 20% of clones from T0, 50% of clones from T2 and 12.5% of clones from T6 of the responder as well as in 33% of clones from T0 and 17% of clones from T6 of the reverter. Both the Asp^{144} to Gly^{144} and the Glu^{164} to Gly^{164} mutations may interfere with recognition of HBsAg by antibodies, therefore showing reduced binding to anti-HB antibodies and could thus represent novel mutations, which have the potential to become vaccine escape mutants.

Mutations occurring in the immunodominant epitope for B-cell recognition (amino acids 178-186), may be caused by negative selection induced by the immune response (Ong *et al*., 2005). Two mutations occurred within this epitope region. Firstly, a Trp^{182} was substituted for a stop codon in 10% of T0, 20% of T2 and 67% of T4 clones from the non-responder. In this patient, IFN seemed to be selecting for mutants capable of evading the immune response, thus resulting in an increase in this mutation over time.

Secondly, in the responder, a Leu^{186} to Pro^{186} mutation occurred in 20% of clones from T0 and T2 and in 37.5% of clones from T6. Leu^{186} is essential for the reactivity of the epitope (Paulij *et al.*, 1999) thus substituting it for a proline would possibly alter the structure of the epitope, thus affecting the binding of the B-cells. Leucine, a non- polar, unreactive, hydrophobic amino acid and proline a non-polar, cyclic amino acid are chemically very different. However, because this mutation was present in the responder at the start and end of treatment, perhaps it does not inhibit B-cell binding but instead enhances it, thus contributing to the increased clearance of HBV-infected cells. This mutation may therefore be detrimental to the virus. Clearly, further studies would be needed to elucidate the exact role, if any, of this mutation on IFN response.

From phylogenetic analysis of the patients at the different times of sampling, it would seem that when an external factor such as IFN therapy is introduced into a system, it may disrupt the balance within that system, thus allowing the loss of original viral strain and the subsequent takeover by a minor population from the quasispecies pool. The reason for this may be that the major population is more susceptible to IFN than the minor strains, thus allowing IFN to enhance the immune response toward the major strain and subsequently causing the levels of the minor strain to increase (Hannoun *et al*., 2002b). However, when the pressure is removed, as was the case at T6, 40 weeks after the initial onset of treatment, the original viral population re-emerges.

When this phylogenetic data is related to the mutations found within the patients, we see the presence of certain mutations in the responder that may represent IFN treatment

escape mutants. Gln^{54} to His^{54} in the preS1 at all time points, Met^{133} to Thr^{133} and Asp^{144} to Gly^{144} at all time points as well as Glu^{164} to Gly^{164} and Leu^{186} to Pro^{186} at T0, T2 and T6 of HBsAg- which may represent the dominant HBV population within this patient. At T4, with the separation into three distinct clusters, we also see the decrease of the Gln^{54} to His^{54} mutation in preS1, the emergence of a Gln^{10} to Arg^{10} mutation in preS2 and as already noted, the disappearance of the Glu^{164} to Gly^{164} and Leu^{186} to Pro^{186} mutations in HBsAg. This variation in amino acids at T4 may therefore account for the emergence of new viral populations.

In the reverter, it would seem that the Gln^{10} to Arg^{10} mutation in preS2, present at all the time points and Gln^{129} to Arg^{129} mutation in HBsAg present at T0, T2 and T6, represent the dominant population in this patient. At T2, there was a separation of the strains into two clusters and we see the emergence of a Gly^{112} to Arg^{112} mutation, which may be responsible for the separation into two clusters. At T4 there was a further splitting of the populations, and the emergence of the Met^{133} to Thr^{133} mutation, also found in the responder, thus explaining why these HBV isolates seem to cluster together at T4.

In the non-responder, the Gln^{10} to Lys^{10} mutation in preS2 was present throughout all time points and represents the dominant population. The Gly^{112} to Arg^{112} mutation, also found in the reverter, may be responsible for the clustering together of the HBV isolates from these patients at T4.

Chapter 5: CONCLUSION

As all strains from the responder, reverter and non-responder were infected with subgenotype A1/serological subtype *adw*2, genotype/serotype differences could not account for differences in response between the patients. There were no differences in amino acid divergences of the complete S ORF or the HBsAg alone between the HBV isolates from the responder, reverter and non-responder. The preS1 region of the responder showed the highest amino acid divergence compared to the non-responder/reverter. On the other hand, the most variable region, the preS2 was least conserved in the non-responder and reverter at all time points and therefore a more conserved preS2 may represent a predictive factor for response to IFN therapy. The responder strains cluster separately from the non-responder/reverter strains at T0. In the responder we see the persistence of a HBV strain that may have IFN treatment "escape" mutations. In the reverter we see the emergence of a new population of virus that clusters in the same branch as the responder strains at times T2 and T4. A new strain that clusters with the isolates from the non-responder was also seen to emerge in the responder at T4, with the original strains re-emerging after the termination treatment in all patients. Contrary to observations of other limited studies on different genotypes (Liu *et al*., 2004), in the present study we see that the viral population dynamics differ between the responder strains and the non-responder/reverter strains. Further research with more cases and functional studies, such as the approach used by Wang *et al* (2005), are therefore needed to determine whether these observations are pertinent only to this pilot study or

may be more generalized phenomenon that may provide us with a predictive tool for IFN treatment of patients infected with subgenotype A1 isolates of HBV.

Chapter 6: REFERENCES

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Appendix A1: ETHICS APPROVAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Gous

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M050705**
PROJECT	A Retrospective Study Characterizing the Complete Hepatitis B Virus Genomes from Black Children....(re-submission M050622)
INVESTIGATORS	Miss N Gous
DEPARTMENT	Molecular Hepatology Res. Unit
DATE CONSIDERED	05.07.29
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 05.08.01 **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

Appendix A2: ETHICS APPROVAL FROM NATAL

UNIVERSITY OF
KWAZULU-NATAL

4 April 2005

Miss N M Gous
Molecular Hepatology Research Unit
University of the Witwatersrand

Fax : 011 643 8777

Dear Miss Gous

PROTOCOL : A prospective study characterizing complete Hepatitis B virus genomes from Black children with membranous nephropathy treated with Interferon alpha 2 beta (α2β). N M Gous, Medicine, Wits. Ref.: E114/04

The Biomedical Research Ethics Committee considered the abovementioned application and the protocol was approved at its meeting held on 25 January 2005 pending queries being addressed appropriately. This condition has now been met, the study is give full ethics approval and may begin as at today's date : 4 April 2005.

This approval is valid for one year from 25 January 2005. To ensure continuous approval, an application for recertification should be submitted a couple of months before the expiry date.

May I take this opportunity to wish you everything of the best with your study. Please send the Biomedical Research Ethics Committee a copy of your report once completed.

Yours sincerely

PROFESSOR A DHAI
Chair : Biomedical Research Ethics Committee

c.c. Professor R Bhimma, Paediatrics.

Appendix A3: PERMISSION FOR USE OF SERUM

Professor R Bhimma
Specialist Paediatrician and Paediatric Nephrologist
Department of Paediatrics and Child Health
Private Bag 7, Congella, 4013 Tel: 031- 260 4351 CELL: 083 799 5674
FAX: 031 – 260 4388

12 May 2004

To Whom It May Concern:

Permission for use of Serum Samples for Genomic Analysis

This is to state that I (Rajendra Bhimma) grant permission to Dr Anna Kramvis and her collaborators to use the serum samples I collected as part of my study on the Efficacy of Hepatitis B virus associated nephropathy (Study No: 125/96) for genomic analysis.

Yours sincerely

R Bhimma

Appendix B: PATIENT'S INFORMED CONSENT

ORMED CONSENT FOR INCLUSION IN A CLINICAL TRIAL§

E.8.1 **I,(Name)**
hereby consent to the following Procedure and/or Treatment being conducted on myself or the person indicated in (iv) below:

E.8.2 **I acknowledge that I have been informed by:**

(Name) DR R BHIMMA et al
concerning the possible advantages and possible adverse effects which may result from the abovementioned procedure and/or treatment and of the ways in which it is different from the conventional procedure and/or treatment.

I,(Name)
hereby acknowledge that I understand and accept the "Information to Patients" leaflet handed to me in connection with this trial.

8.3 **I agree that the above procedure and/or treatment will be carried out and/or supervised by**

(Name) DR R BHIMMA et al

8.4 **I acknowledge that I understand the contents of this form, including the information provided in the "Information to Patients" leaflet and as the**

☐ ***SUBJECT** ☐ **PARENT** ☐ **GUARDIAN** ☐ **OTHER (specify)**

freely consent to the above procedure and/or treatment being conducted on:

(Name)

8.5 **I am aware that I may withdraw my consent at any time without prejudice to further care.**

Signed: _____ Date:
Subject/Parent/Guardian

Signed: _____ Date:
Witness

Signed: _____ Date:
Informant

Signed: R Bhimma Date:
Researcher

§ With the exception of the names and signatures in paragraphs 8.1, 8.4 and 8.5, please provide the above information.

Appendix C: CHANGE OF TITLE

Faculty of Health Sciences
UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
7 York Road PARKTOWN Johannesburg 2193 Telegrams WITSMED Telex 4-24655.SA
FAX 643-4318 TELEPHONE 717-2075/2076
E-MAIL healthpg@health.wits.ac.za

MS NM GOUS
8 WILDEBEES STREET
RANT EN DAL
KRUGERSDORP
1739

APPLICATION NUMBER 0008280D
STATUS (DEG 15) (MM041) TAA

2005-09-27

Dear Ms Gous

Change of title

I am pleased to inform you that the following change in the title of your dissertation for the degree of Master Of Science In Medicine (Full-Time) has been approved:

From:
A retrospective study characterzing the complete S open reading frame of Hepatitis B virus from black children with membranous nephrology treated with Interferon X2b

To:
A retrospective study characterzing the complete S open reading frame of Hepatitis B virus from black children with membranous nephropathy treated with Interferon alpha 2b

Yours sincerely

S Benn (Mrs)
Faculty Registrar
Faculty of Health Sciences

Plan your career and first-year curriculum online - explore our website: www.wits.ac.za/ec2
The University seeks to serve South Africa by furthering access to equal opportunity while striving for excellence in

Appendix D: SOLUTIONS, CHEMICALS AND REAGENTS

D1. Solutions

0.8% Agarose Gel – Crystal Violet

- 0.4 g Molecular Grade Agarose
- 50 ml 1X TAE Buffer
    - Heat in microwave for approximately 3 minutes or until agarose is dissolved;
    - Cool to ~ 55º C;
    - Add 45 µl of 2 mg/ml crystal violet and swirl to mix;
    - Pour into gel tray with comb and allow to set.

1% Agarose Gel – Ethidium Bromide

- 1,5 g Molecular Grade Agarose
- 150 ml 1X TBE Buffer
    - Heat in microwave for approximately 3 minutes or until agarose is completely dissolved;
    - Cool to ~ 55º C;
    - Add 20 µl ethidium bromide and mix;
    - Pour into gel tray with comb and allow to set.

3% Composite Agarose Gel – Ethidium Bromide

- 6 g NuSieve GTG Agarose
- 3 g Molecular Grade Agarose
- 300ml 1X TBE Buffer
    - Heat in microwave for approximately 5 minutes or until boiling, stirring occasionally;
    - Cool to ~ 55º C;
    - Add 30 µl ethidium bromide and mix;
    - Pour into gel tray with comb and allow to set.

Bromophenol Blue Loading Dye

- 0.01 g Bromophenol blue
- 2 ml 0.2 M EDTA
    - 0.4 ml EDTA
    - 1.6 ml BQW
- 5 ml 50% glycerol
    - 2.5 ml glycerol
    - 2.5 ml BQW
    - Make up to 10 ml with distilled water and mix;
    - Aliquot into 1.5 ml microcentrifuge tubes;
    - Store at 4ºC.

Ethidium Bromide Stock (10 mg/ml)

- 0.1 g Ethidium Bromide
- 10 ml Best quality water
    - Mix well;
    - Store in a dark bottle at 4º C.

Kanamycin Stock

- 0,5 g Kanamycin
    - Make up to 10 ml with Best Quality Water;
    - Shake well to mix;
    - Filter sterilize;
    - Aliquot into 1.5 ml microcentrifuge tubes;
    - Store at 4º C.

Luria-Bertani (LB) Medium

- 10 g Tryptone
- 5 g Yeast Extract
- 10 g Sodium Chloride
    - Make up to 1 L with distilled water and stir;
    - Autoclave;
    - Store at 4º C.

Luria-Bertani (LB) agar plates with Kanamycin

- 10 g Tryptone
- 5 g Yeast extract
- 10 g Sodium chloride
- 20 g Bacteriological agar
    - Make up to 1 L with distilled water and stir;
    - Autoclave;
    - Cool to ~ 55º C;
    - Add 1 ml of 50 µg/ml of kanamycin antibiotic;
    - Pour into plates;
    - Allow to set, invert plates and then store at 4º C.

TAE Buffer (10X)

- 24,2 g Tris-base
- 5,68 ml Glacial acetic acid
- 10 ml 0.5 M EDTA (pH 8)
    - Make up to 1L distilled water;
    - Autoclave;
    - Store at room temperature.

TBE Buffer (10X)

- 108 g Tris-base
- 9.3 g EDTA
- 55 g Boric acid
    - Make up to 1L with distilled water;
    - Autoclave;
    - Store at room temperature.

D2. Chemicals and Reagents

All chemicals and reagents used for this project as well as their sources are listed in Table A1 below.

Table A1: Chemicals and Reagents

Chemicals and Reagents	Manufacturers
Acetic acid glacial	Associated Chemical Enterprises
Bacteriological agar (European formulation)	Whitehead Scientific
Boric acid	Sigma
Bromophenol blue	Merck
Ethanol (99.7 – 100%)	Saarchem, Mercke
Ethidium Bromide	Sigma
Ethylenediamine tetra-acetic acid di-sodium salt dehydrate (EDTA)	Saarchem, Mercke
Glycerol	Saarchem, Mercke
Hydrochloric acid	Saarchem, Mercke
Kanamycin Sulphate	Roche
Molecular grade agarose	Bioline
NuSieve GTG Agarose	Cambrex

Sodium Chloride (NaCl)	Saarchem, Mercke
Tris[hydroxymethyl]aminomethane	Roche
Tryptone (Pancreatic digest of casein)	Pronadisa
Yeast extract	Pronadisa